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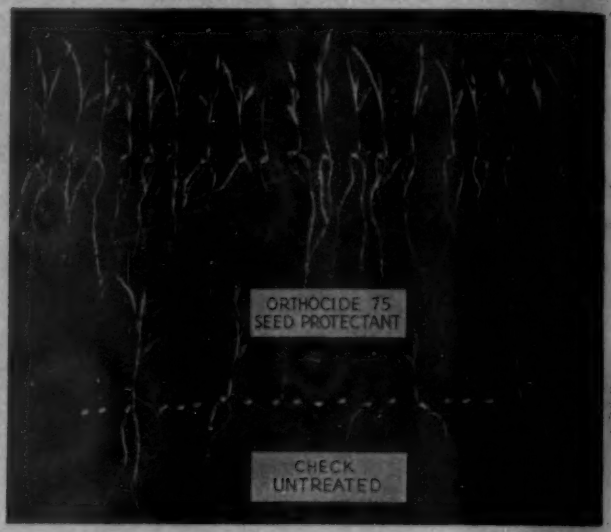

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CLAYTON ROBERTS ORTON, 1885–1955

J. G. Leach and Frank D. Kern

Clayton Roberts Orton, Professor of Plant Pathology at West Virginia University, died June 17 in Pittsburgh, just 13 days before anticipated retirement.

Dr. Orton was born in East Hardwick, Vermont, April 1, 1885. He received the B.S. degree at the University of Vermont in 1909, the M.S. at Purdue University in 1915, and the Ph.D. from Columbia University in 1924. In 1942, the University of Vermont conferred upon him the honorary degree of Sc.D.

He began his professional career as Special Agent in the U. S. Department of Agriculture in 1909. The next year he was Assistant Plant Pathologist at the University of Wisconsin, then for three years he was Assistant Botanist at the Purdue University Agricultural Experiment Station. From 1913 to 1927 he was a member of the staff of the Department of Botany and Plant Pathology, the Pennsylvania State College, serving in turn as Assistant Professor, Associate Professor, and Professor.

He went to West Virginia University in 1929, after serving for 4½ years as Plant Pathologist for the Bayer Semesan Company. At West Virginia, he served as Head of the Department of Plant Pathology (1929-33), Head of the Department of Biology (1933-36), and Head of the Department of Plant Pathology and Forestry (1936-38). In 1938 he was made Dean of the College of Agriculture and Director of the Agricultural Experiment Station. While serving in this capacity, he was instrumental in many progressive developments, including the establishment in 1938 of the Division of Forestry, which became a fully accredited school in 1948. A Department of Agricultural Engineering was organized under his guidance.

In 1949 he relinquished his administrative duties and returned to the Department of Plant Pathology as Research Professor. From 1950 to 1953 he was on leave while he served as Director of Research and Extension in Agriculture for the Technical Cooperation Administration in Monrovia, Liberia. Here he set up the first full-scale Point IV Agricultural Program and organized and put into operation a staff of 16.

Dr. Orton's service to West Virginia went beyond his duties as Department Head and Dean and Director. He was deeply interested in all aspects of West Virginia agriculture, especially conservation of soil and water. For 15 years he served as a member of the West Virginia State Planning Board and was its chairman from 1941 until his death. He served many years as a member of the Technical Advisory Committee and alternate delegate to the Potomac River Basin Commission. For eight years, he was Secretary of the Experiment Station Section of the Association of Land Grant Colleges and Universities and was chairman of the section in 1948.

On September 22, 1911, he married Ethel M. Chapman, whose social charm and friendly interest in her husband's students and associates contributed much to his professional success. Besides his wife, he is survived by a son, Gardner, and two daughters, Mrs. Jean Rhys and Mrs. Patricia Spurr.

CLAYTON ROBERTS ORTON, 1885–1955

Dr. Orton was a faithful member of the Episcopal church and a leader in many local welfare activities. He was active in numerous professional societies and was elected President of the American Phytopathological Society in 1939. He was a member of several honorary scientific organizations, including Sigma Xi, Alpha Zeta, Gamma Sigma Delta, and Phi Epsilon Phi.

Dr. Orton was author or co-author of more than 100 professional papers dealing with a variety of subjects. In the fields of plant pathology and mycology, he is best known for his work on potato diseases, seed-borne diseases, the plant rusts, and the genus *Phyllachora*. After retiring from administrative duties, he had renewed his interest in the species of *Phyllachora* affecting grasses, and at the time of his death had almost completed a paper on the morphology and life history of *Phyllachora punctatum*.

Dr. Orton's death will be felt as a great loss by the department of which he was member, by his professional associates, and by a host of friends.

PHYTOPATHOLOGY for April (46: 187-242) was issued April 20, 1956

INSECT TRANSMISSION OF OAK WILT [1]

Frederick F. Jewell [2]

SUMMARY

Experiments made during 1953 and 1954 demonstrated that artificially contaminated nitidulids caged on suitable wounds are capable of bringing about infection of healthy oaks. The species of Nitidulidae used in successful inoculations were *Glischrochilus sanguinolentus* (Ol.), *G. confluentus* (Say), *Colopterus semitectus* (Say), and *Carpophilus lugubris* Murr. Other insects, including species of the families Scolytidae, Histeridae, and Formicidae, and *Drosophila* spp., were used in some experiments, but they did not transmit the disease. All positive cases of insect transmission resulted from inoculations made in May and early June. Those made in summer and early fall were negative.

Insects were quickly attracted in large numbers to fresh wounds made on oak trees during late April and throughout May, but few were attracted to such wounds during the summer months. Species of Nitidulidae were the insects found most abundant in the wounds. Fresh wounds, with the bark loosened from the sapwood, made in the spring were most attractive to insects, and they remained attractive 2–3 weeks. *Graphium rigidum* (Pres.) Sacc. and *Ophiostoma pluriannulata* (Hedg.) Syd. were constantly found associated with nitidulids in wounds in healthy trees where these fungi served as a source of food for the insects. Nitidulid eggs and larvae were found in many wounds, where the larvae completed most of the their development. Evidence was obtained that pupation of *Colopterus morio* (Er.), *C. semitectus*, and probably other species of nitidulids occurs in the duff at the base of wounded trees. Inoculations made in April with spore suspensions sprayed on wounds similar to insect-infested wounds and like those used in the insect transmission experiments were effective, showing that such wounds are suitable infection courts.

Species of Nitidulidae were found in all cases to be the most numerous insects associated with the mycelial mats. Many species of insects present on the mats were the same as those found in fresh wounds. Nitidulid adults and larvae were observed feeding on the fungus. No nitidulid pupae were found on any of the mats examined, but a few were recovered from the duff at the base of the trees.

The heterothallic nature of *E. fagacearum* was utilized to develop a technique for demonstrating the presence of spores of the oak wilt fungus on nitidulids found on diseased trees. By means of this technique, the oak wilt organism was demonstrated in the fecal pellets of several species of nitidulids. Fecal pellets from culture-fed nitidulids, when suspended in sterilized water, served as effective inoculum when injected into fresh wounds. The value of the spermatizing technique in detecting the presence of the oak wilt pathogen in mixed cultures of fungi was demonstrated.

Evidence was obtained of an apparently symbiotic relationship between the Nitidulidae and *G. rigidum* and *O. pluriannulata;* the fungi are disseminated by the nitidulids, which in turn receive nutritional benefits from the 2 fungi. The theory is advanced that these 2 fungi are the original fungus members of the symbiotic relationship and that the oak wilt fungus is a relatively recent member. If this theory is correct, the association with *E. fagacearum* can be expected to become more general as time passes, with a corresponding increase in rate of spread of the disease. This probable increase in the association of *E. fagacearum* with the Nitidulidae further justifies efforts now being made to bring the disease under control as promptly as possible.

Oak wilt was first reported by Henry and Moses (19) in 1943. In 1944, Henry et al (20) published a more complete description of the disease, and on the basis of the imperfect stage, Henry (18) named the pathogen *Chalara quercina*. Bretz (8) in 1951 obtained fertile perithecia when different isolates were grown together in culture and renamed the fungus *Endoconidiophora fagacearum* (9).

Oak wilt was first found in West Virginia in 1951, when a preliminary survey revealed 54 diseased trees in 3 separate regions within the state (37). At this time the only known method of spread of the disease was through naturally occurring root grafts as described by Kuntz and Riker (26).

Curl et al (12) were the first to observe the fructification of the pathogen in nature. Large macroscopically visible mats were found between the bark and wood of the diseased trees in Illinois forests during August and September 1951. Similar mycelial mats developed when bark samples from diseased trees were stored in a moist closed box. The pressure cushions and their function in cracking the bark apparently were not recognized at this time. The mycelium was observed growing in the channels of several wood-boring insects, but no functional relationship between the insects and the fungus was reported. In May 1952 Staley and True (35) and Leach et al (30) observed the mats in various stages of development and described the mechanism by which pressure cushions formed by the pathogen cracked the bark

[1] Accepted for publication December 4, 1955.
Published with the approval of the Director of the West Virginia Agricultural Experiment Station as Scientific Paper No. 513.

In this paper the word transmission has been used in the sense outlined by Leach (27, p. 593).

[2] Formerly Assistant in Plant Pathology at West Virginia University. Now Plant Pathologist, Southern Institute of Forest Genetics, U. S. Forest Service, Gulfport, Mississippi.

and exposed the sporulating mycelium (Fig. 1, 2, 3). They pointed out that the freshly exposed mycelial mats formed only endoconidia but that later, after the mats had been visited by insects, abundant fertile peri-

FIG. 1–2. Fig. 1. A crack in the bark of an oak tree killed by wilt. The crack, which was caused by the pressure exerted by the pair of superimposed pressure cushions formed by the pathogen between the bark and sapwood, exposes the fungus mat to insects attracted by its characteristic odor.—Fig. 2. A mycelial mat and the pairs of pressure cushions responsible for the cracking of the bark. The left half shows the surface of the sapwood; the right half, the inner surface of the bark and the crack through which the nitidulids reached the mat. The mat had been spermatized and the nitidulids were feeding on the ascospores.

thecia developed. Barnett et al (4), using the method of spermatizing cultures described by Hepting et al (22), demonstrated that perithecia would form on freshly exposed mats within 3 days after spermatization. True et al (38) observed that several species of sap-feeding beetles (Nitidulidae) were attracted by the characteristic odor of the fungus to the subcortical mycelial mats as soon as the bark-cracks were

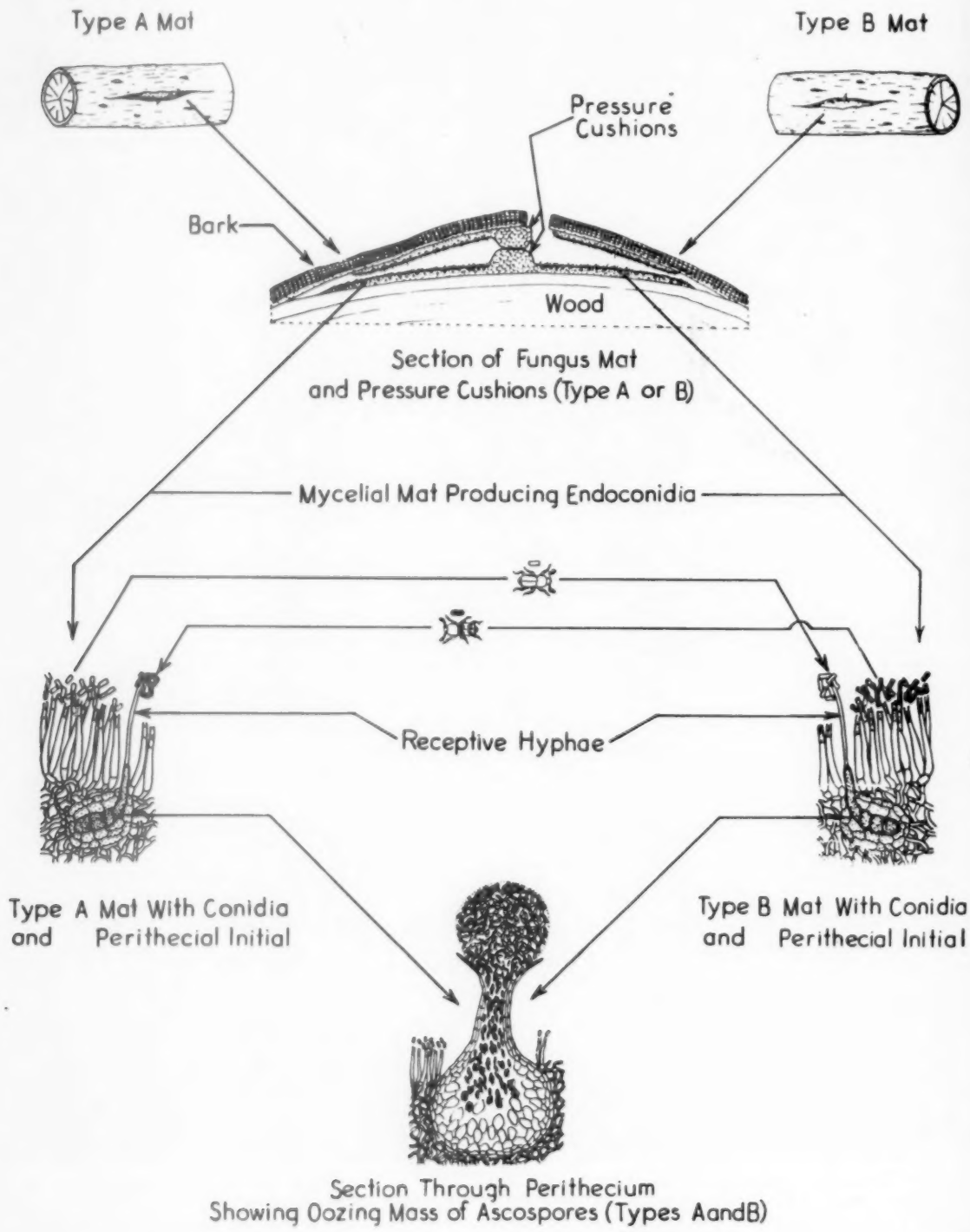
Type A Mat
Type B Mat
Pressure Cushions
Bark
Wood
Section of Fungus Mat and Pressure Cushions (Type A or B)
Mycelial Mat Producing Endoconidia
Receptive Hyphae
Type A Mat With Conidia and Perithecial Initial
Type B Mat With Conidia and Perithecial Initial
Section Through Perithecium Showing Oozing Mass of Ascospores (Types AandB)

FIG. 3. A diagrammatic representation of the relationship between nitidulids and the sexual reproduction of *Endoconidiophora fagacearum*.

formed. They demonstrated that beetles from a mat of one compatibility type would transmit the spores necessary for spermatizing the mat mycelium of the opposite type and induce perithecia formation (Fig. 3). These insects were thus shown to be agents of spermatization, and it was predicted (28, 30) that by suitable experiments they would be found also to be vectors of the disease. Later, Morris and Fergus (33) observed mats in Pennsylvania and pointed out their importance as sources of inoculum.

Insect transmission experiments in which several species of Nitidulidae and Scolytidae were used were begun in West Virginia in 1952. The scolytids had been trapped as they emerged from oak trees previously killed by wilt. Nitidulids had been obtained from oak wilt mats and from wounds. Only negative results were obtained from a total of 72 trees of the red oak group on which these insects were caged during the spring, summer, and fall of 1952 (13).

The investigations reported here were begun in 1952 and completed in the fall of 1954. These studies were carried out to determine 1) if oak wilt could be transmitted in nature by insects, 2) what insect or group of insects was chiefly responsible, and 3) the nature of the relationship of the vectors to the pathogen.

INSECT INOCULATION EXPERIMENTS. — *Transmission studies, 1953.*—The 1953 inoculation experiments were made on pole-size red and black oaks in an area free of oak wilt. In the first part of this work the bark of a trunk at breast height was smoothed with a drawknife. Care was taken not to cut into the inner bark or sapwood. When nitidulids were used, a slanting chisel cut was made at the center of the smoothed area, penetrating into the sapwood to a depth of ½–1 in. The bark around the wound was then loosened. The nitidulids were contaminated by allowing them to feed on petri dish cultures of *E. fagacearum;* some cultures contained both ascospores and conidia and others only conidia. Beetles were placed in cellulose acetate vials (1 × 4 in.) fitted with plastic collars by which they were attached to the trees (Fig. 8, 9). In later experiments, inoculations were made on larger blaze-type wounds. These were made by completely removing a piece of bark, thus exposing the sapwood, and then loosening the bark around the edge of the wound. A circular plastic screen cage (Fig. 9) was constructed and attached over the wound by means of cardboard collars. Some of the wounds were not used immediately after they were made. In many instances, nitidulids were found under the loosened bark of such wounds before they were caged. These beetles apparently had been attracted by the odor of the fresh wound, and their presence indicated that such wounds were likely infection courts in nature.

Some of the insects used in these experiments were obtained from bait traps. Others came from fresh wounds on healthy oaks. The following species of Nitidulidae were used: *Glischrochilus confluentus* (Say), *G. fasciatus* (Ol.), *G. sanguinolentus* (Ol.), *Carpophilus lugubris* Murr., and *Colopterus semitectus* (Say). Other insects used in a few inoculations included members of the families Formicidae and Scolytidae and of the dipterous genus *Drosophila.* The Scolytidae did not penetrate the bark or wood of the healthy trees and died very quickly, as did the ants and *Drosophila* spp. Several variations in inoculation methods utilizing different types of insect cages were tried in attempts to increase insect longevity and penetration, but none was successful.

Because of the lack of suitable numbers of insects during the summer, no definite inoculation schedule was maintained; however, 10–15 insects were utilized per tree whenever possible. Six trees were inoculated on May 6 with 3 species of Nitidulidae: *C. semitectus* (Fig. 13), *G. sanguinolentus* (Fig. 14), and *G. confluentus.* On May 8, 5 additional trees were inoculated by means of the nitidulids *G. confluentus, G. fasciatus, C. lugubris* (Fig. 15), and *C. semitectus. C. lugubris* was used in combination with each of the other species on separate trees. *Drosophila* spp. were used on 2 of the trees inoculated. These 11 inoculations were the only ones in 1953 in which chisel cuts were utilized. The remainder of the 1953 inoculations employed other kinds of wounds, mainly a blaze-type.

Inoculated trees were inspected weekly. On June 12, 5 of the 6 trees inoculated by means of nitidulids on May 6 and one of those inoculated on May 8 had typical oak wilt symptoms. Branch samples taken from each of these trees and cultured on glucose-phenylalanine agar (1) yielded *E. fagacearum* (Table 1). Since the only source of inoculum in these experiments was

TABLE 1.—*Evidence of infection of single oak trees inoculated by means of spore-carrying Nitidulids placed in chisel cuts*

Insect species	No. of insects used	Type of inoculum[a]	Symptoms[b]	Culture results[c]
(Inoculated May 6)				
Colopterus semitectus and *Glischrochilus sanguinolentus*	10	C	+	+
G. sanguinolentus	20	C	+	+
G. confluentus	10	C	—	—
C. semitectus	10	C & A	+	+
C. semitectus	14	C & A	+	+
C. semitectus	10	C & A	+	+
Drosophila sp.	21	C	—	—
(Inoculated May 8)				
G. confluentus and *Carpophilus lugubris*	8	C & A	+	+
Drosophila sp.	4	C	—	—
C. lugubris and *C. semitectus*	16 11	C	—	—
C. lugubris and *G. fasciatus*	20 10	C & A	—	—

[a] Before they were placed in wounds, the insects had fed on cultures of *E. fagacearum* bearing either conidia (C) or conidia and ascospores (C & A).

[b] + Indicates that the tree had typical oak-wilt symptoms on June 12; —, that no oak-wilt symptoms developed.

[c] + Indicates that branch samples cultured on glucose-phenylalanine agar yielded *E. fagacearum;* —, that the fungus was not recovered.

the spores of the fungus carried by the insects, these results show that the species of Nitidulidae used in this study can serve as vectors of oak wilt.

Transmission studies, 1954.—Additional transmission experiments were made in the same area during the spring and early summer of 1954. Each tree of the red oak group selected for transmission studies was located at least 100 ft. from any previously inoculated tree. The insects used were taken from bait traps, from fresh wounds, or from mycelial mats on trees killed by oak wilt. The insects, except those from mats, were fed on conidial cultures.

A different type of insect cage was used in 1954 in an effort to reduce the mortality rate of the experimental insects. A flat cage was designed to allow the insects more freedom of movement over the surface of the wound, at the same time keeping them closer to the infection court. This cage consisted of 5×10-in. rectangular piece of unbleached muslin folded once. A hole was cut in the center of the 2 halves and a

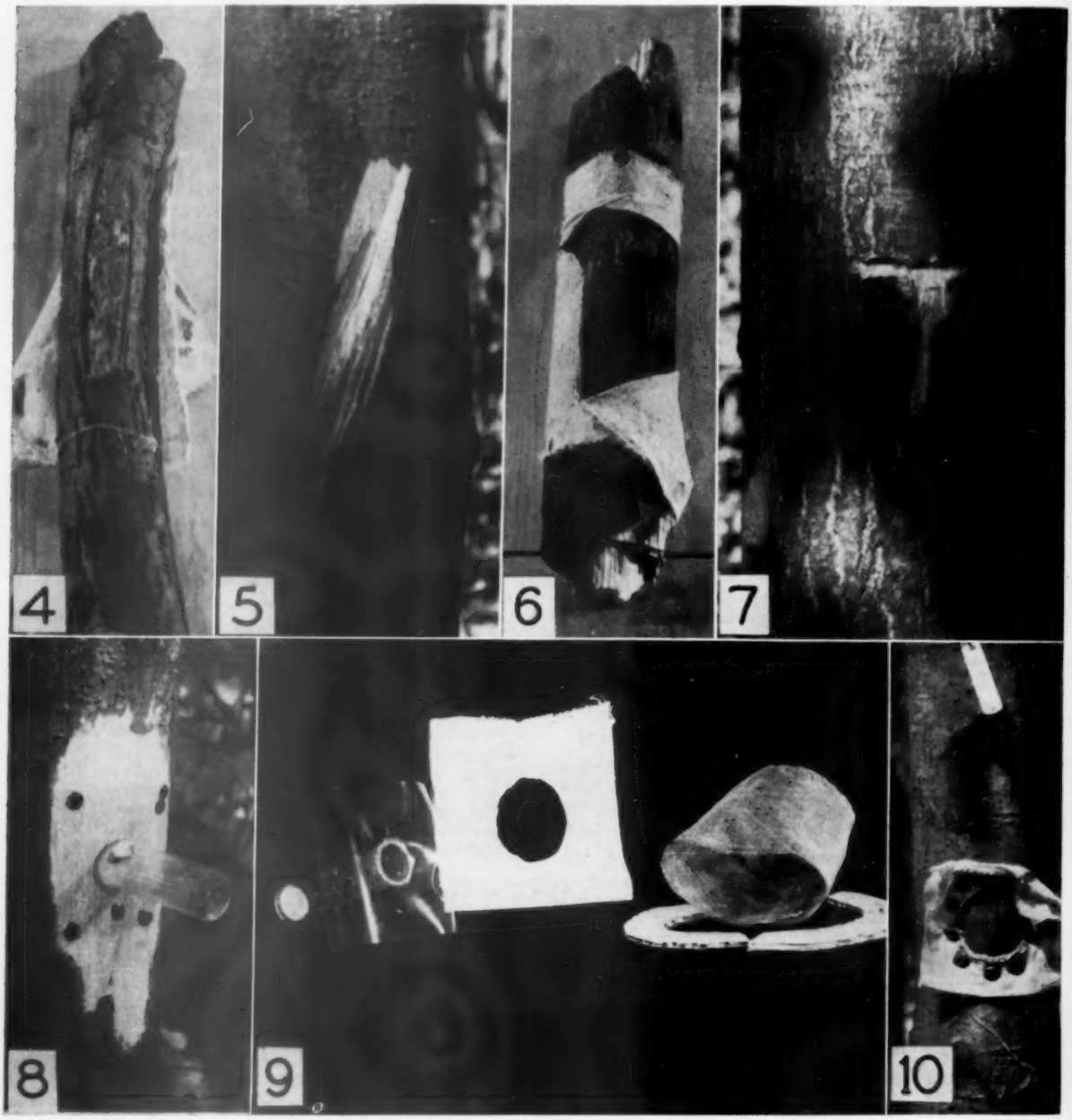

FIG. 4–10. Four types of wounds and 3 types of cages used in insect-transmission experiments.—Fig. 4. Blaze wound.—Fig. 5. Slash wound.—Fig. 6. Bruise wound.—Fig. 7. T-shaped wound.—Fig. 8. A celluloid-vial cage in position.—Fig. 9. The 3 types of cages.—Fig. 10. A flat plastic-screen cage in position.

piece of fine-mesh screen stapled over the hole with its edges between the 2 folds of the cloth (Fig. 10).

Several types of wounds other than the chisel-type wounds were utilized in these 1954 experiments. The types used were termed blaze, slash, bruise, and T-shape (Fig. 4–7).

The 1954 transmission experiments were carried out from April 10 to June 10. Insofar as possible, inoculations were made weekly during this period. None of the trees inoculated in April became infected. Only 1 of several trees inoculated in May developed oak wilt. This tree was inoculated on May 29 by placing 10 individuals of *C. lugubris* on a slash-type wound.

Two trees were inoculated on June 10 by means of *C. lugubris*. One of these developed oak wilt symptoms about 6 weeks after inoculation. Samples were taken from each tree showing oak wilt symptoms, and infection was verified by laboratory culture.

The April inoculations with insects were earlier than any previously made, the trees selected having no open leaf buds. Since it was not known whether trees inoculated with spore suspensions at this stage would

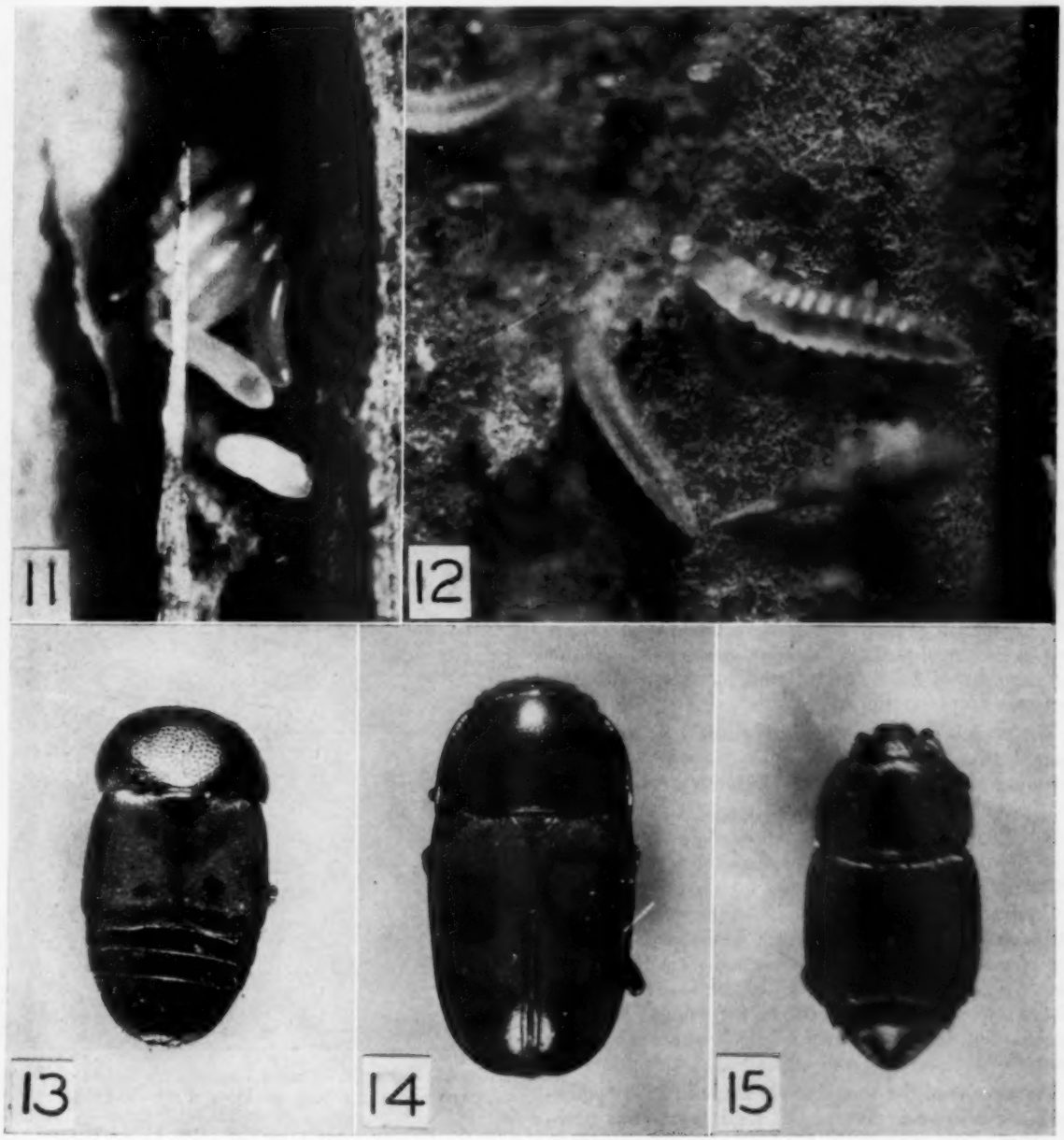

FIG. 11–15. Fig. 11. Nitidulid eggs.—Fig. 12. Nitidulid larvae on mycelial mats of *E. fagacearum* in nature.—Fig. 13–15. Three species of nitidulids that transmitted oak wilt under experimental conditions.—Fig. 13. *Colopterus semitectus* (Say).—Fig. 14. *Glischrochilus sanguinolentus* (Ol.).—Fig. 15. *Carpophilus lugubris* Murr.

develop the disease, a parallel series of inoculations using spore suspensions was made on April 24 about 4 miles from the area used for the insect-inoculation tests. The size of the trees and the types of wounds used were similar in the 2 tests. Five red oaks were inoculated with a conidial suspension applied with an atomizer to the surface of the wound. The wound was then tightly covered with a piece of muslin. All 5 trees developed typical oak wilt symptoms in about 6 weeks. Cultures made from branch samples were positive for all 5 trees. Obviously the negative results obtained with the insect inoculations made at the same time were not due to a lack of susceptibility.

INSECTS ASSOCIATED WITH WOUNDS.—Leach et al (30) and Craighead et al (10) pointed out that nitidulid beetles were attracted to wounds in healthy oaks. Studies of the attractiveness of wounds to insects were begun about May 1, 1955. The wounds used were of no specific shape or size, but all had a large area of exposed sapwood, around the edges of which the bark was separated somewhat from the wood. At this time, the bark was quite easily separable from the moist sapwood (Fig. 16).

Four or 5 days after the wounds were made, as many as 50 nitidulids were found under the loosened bark of a single wound. The principal species collected were *C. lugubris* and *C. semitectus*. Most of them were collected during the first 3 weeks of May, although some were collected in June.

The wounds remained attractive to adult nitidulids for 2–3 weeks. In general, they ceased to be attractive when the exposed sapwood and the wood under the loosened bark became dry. At that time, the adult nitidulids were no longer present in the wounds, but numerous larvae in all stages of development were found. At this time, both empty egg shells and freshly deposited insect eggs were observed on the wounds. The unhatched eggs were collected, taken to the laboratory, and incubated. Nitidulidae larvae hatched from the eggs, but did not survive until maturity. The species was not determined.

The presence of the adult nitidulids, their eggs, and their larvae indicated that such wounds served as breeding places. No pupae of nitidulids were observed on the wounds. It was assumed that the larvae, after completing most of their development on the wounds, migrated to the duff at the base of the tree on which they were found.

Predaceous insects appeared and preyed on the larvae soon after the nitidulid eggs hatched. The families of predaceous insects represented were Histeridae, Staphylinidae, Cucujidae, and Colydiidae. The species of predaceous insects present in the wounds were recorded, but the exact time of their appearance was not noted.

The results of the wound study made in 1953 emphasized further the importance of fresh wounds as probable infection courts for the oak wilt fungus when transmitted by insects. To determine more definitely what species of Nitidulidae were attracted to wounds and at what time during the year such wounds were most attractive to insects, another wound study was started in February in 1954.

Trees of the red oak group were wounded each month until August. The wounds were of 2 types: blaze and bark bruise (Fig. 4, 6). These were made at either breast height or at the base of the trees. The bark around the blaze-type wounds was loosened in all cases. When insects were collected from a wound, care was taken not to enlarge the original wound. This made the collection of insects rather difficult and some insects present were missed, but it is believed that a representative sample of the insect population was taken.

From the observations made during this period, it would appear that wounds made on healthy oaks during late April and throughout May were more attractive to insects, mainly Nitidulidae, than wounds made earlier or later in the year. April and May wounds were attractive to insects for about 3 weeks. The loosened bark and moist under-lying sapwood present during this time of year appear to be the most important factors in attracting insects to such wounds. Wounds made at this time appear to serve as breeding places for many species of Nitidulidae. Eggs and larvae of nitidulids were observed before and after the complete disappearance of the adults from the wounds —about 3 or 4 weeks. Later in the summer, the bark

FIG. 16. An artificial wound made near the base of a red oak tree about the first of May. Five days later it was infested with numerous nitidulids, and there was a dense growth of dark mycelium of *Graphium rigidum* and *Ophiostoma pluriannulata* under the loosened bark. The wound has been enlarged to expose additional fresh sapwood.

of freshly made wounds was not easily loosened. Very few insects were attracted to these wounds. The insects collected from wounds during 1953 and 1954 are listed in Table 2.

On August 11, the duff at the base of trees on which larvae had been collected from wounds in July was examined. At the base of 1 tree, 4 larvae were found at a depth of 2–3 in. These were collected and incubated. Three of these entered the pupal stage 1 day later, apparently without going through another larval instar. The adults emerged in 2–3 days and were identified as *C. semitectus*. At this same time, the duff at the base of another tree was examined, and larvae were again found along with 2 newly emerged adults of *C. morio*. These apparently had just emerged from the pupal stage, as they were almost white instead of the normal black. The larvae immediately began to pupate, and adults emerged from these pupae 3 days later. These also were identified as *C. morio*.

The presence of the larval forms and of newly emerged adults of *C. morio* and *C. semitectus* in the duff is significant in that the place of pupation of many of the Nitidulidae had been in doubt. Even though the pupal stage of these 2 species was not found, the occurrence at the same time of probably the last larval instar form of this species along with newly emerged adults of *C. morio* in the duff at the base of wounded trees is believed to be a good indication that these species pupate in such an environment. It is not unlikely that many of the other nitidulids also pupate in the forest duff.

TABLE 2.—*Insects attracted to wounds and oak wilt mats*

Family	Species	Wounds[a] 1953	Wounds[a] 1954	Mats[a]
Nitidulidae	*Glischrochilus fasciatus*	+	0	+
	G. sanguinolentus	+	0	+
	G. confluentus	0	0	+
	Carpophilus lugubris	+	+	+
	C. corticinus	0	+	
	Colopterus truncatus	+	+	+
	C. morio	+	+	+
	C. semitectus	+	+	+
	Cryptarcha ample	+	+	0
	Stelidota geminata	+	0	0
	Lobiopa undulata	+	+	0
	Epuraea spp.	+	+	+
	Carpophilus spp.	+	+	+
Ostomidae	*Tenebroides* sp.	+	0	+
Histeridae	*Paromalus* sp.	0	0	+
	Platystoma sp.	+	+	+
	Hister sp.	+	+	0
Cucujidae	*Silvanus* sp.	+	+	0
Staphylinidae	*Philonthus* sp.	+	+	+
Scolytidae	*Monarthrum mali*	+	0	+
	M. fasciatum	0	0	+
	Xyloterinus politus	0	0	+
Brentidae	*Arrhenodes minuta*	+	+	0
Curculionidae	*Acalles carinatus*	0	+	0
	Conotrachelus anaglypticus	+	+	0
	Cossonus concinnus	0	0	+
Erotylidae	Unidentified	+	0	0
Colydiidae	*Bitoma* sp.	0	0	+
	Brontis sp.	0	0	+
	Laemophloeus sp.	0	+	+
	Cerelon sp.	0	0	+
Tenebrionidae	*Helops* sp.	0	+	0
Mycetophagidae	*Mycetophagus* spp.	0	0	+
Serropalpidae	*Serropalpus* sp.	0	0	+

[a] + Indicates that insects were collected; 0, that no insects were collected.

Two species of Nitidulidae, *C. lugubris* and *C. semitectus*, were the most abundant insects found inhabiting the wounds during 1953 and 1954. Of these, *C. lugubris* was the more abundant. These species have been used successfully in transmitting oak wilt to healthy oak trees. It is significant that the time when these insects are most abundant in fresh wounds (May) is the time when oak wilt has been experimentally transmitted most successfully by nitidulids. Wounds similar to those from which insects were collected in large numbers were shown to be highly susceptible to infection at the same time of year through the use of spore-suspension inoculations. Also, experimental insect transmission was successful when similar wounds were used as infection courts.

FUNGI ASSOCIATED WITH NITIDULIDS IN FRESH WOUNDS ON HEALTHY TREES.—Nitidulids were most abundant in the artificial wounds on healthy trees when a dark superficial fungus growth was present under the loosened bark. Adults and larvae of the Nitidulidae were observed feeding on this growth. In many cases, heavy sporulation of the fungi consisted of white spore masses plainly visible to the naked eye. Isolations made from samples taken from these wounds constantly yielded 2 fungi, *Graphium rigidum* and *Ophiostoma pluriannulata*. As these fungi produce spores in a sticky matrix, it is reasonable to conclude that the spores were not wind-borne but were introduced into the wound by the nitidulids. The moist conditions present between the bark and sapwood apparently provided a suitable environment for their growth. Figure 16 illustrates a wound with the loosened bark removed, showing the dark mycelial growth present in contrast to the bright sapwood under freshly loosened bark.

The close association of the Nitidulidae with *G. rigidum* and *O. pluriannulata* is significant in that the relationship appears to be of a symbiotic nature. These fungi are not known to be pathogenic, but are known to grow saprophytically on the surface of moist wood. In this study, they were found in wounds only where adult nitidulids were found or had been present. The adult and larval nitidulids frequently were observed feeding on the mycelium and spores of these fungi. Observations indicate that the larvae complete most of their development on the mycelium and then move to the duff at the base of the wounded trees, where they pupate. Whether the fungi are essential for normal development of the insects is not known, but they are utilized as food by both adults and larval nitidulids.

In addition to the artificial wounds examined in this study, several natural wounds resulting from a severe windstorm in the vicinity of Morgantown were examined. These wounds were found inhabited by the

same species of nitidulids found on the experimental wounds. The same 2 nonparasitic fungi also were found growing in the natural wounds.

These same fungi frequently were found growing and sporulating on insect-infested mycelial mats on trees killed by oak wilt. Observations indicated strongly that *G. rigidum* and *O. pluriannulata* were brought to the oak wilt mats by nitidulids.

INSECTS ASSOCIATED WITH THE MYCELIAL MATS OF THE OAK WILT FUNGUS.—Numerous mycelial mats were examined in various parts of West Virginia throughout the year. In all cases, species of Nitidulidae were the most abundant insects present, and they frequently were observed actively feeding on the mycelium. The Coleopterous insects found commonly associated with the mycelial mats are listed in Table 2. Many of the nitidulids found on the mats were of the same species found on fresh wounds. The presence of the same species on both mats and wounds is further evidence that these nitidulids are the most frequent vectors for the oak wilt fungus.

In many instances, fertile perithecia were found on the mats infested with insects. Fertile perithecia result either from the introduction of suitable spores onto the mat or from the presence of the spores or mycelium of the 2 compatibility types intermingled in the same tree, as postulated in an early report by Hepting et al (21). Evidence (2, 3, 7, 14) indicates that in a majority of the cases mycelium of only 1 compatibility type of the oak wilt fungus is found in diseased trees. These facts strongly indicate that perithecia found on mats on wilt-killed trees are most often the result of the introduction of suitable spores from another source onto the mycelium of mats exposed through the cracking of tree bark (Fig. 1, 2) as reported by Leach et al (30) and by Barnett et al (4). That spermatization is usually brought about by insects present on the mats is further indicated by the fact that fertile perithecia have been found in nature only on insect-infested mats.

Eggs and larvae of Nitidulidae (Fig. 11, 12) are common on recently exposed mats.

The observations made on the insect populations of mats provide further evidence that species of Nitidulidae should be considered as the primary vectors of oak wilt. Other insects found on the mats and in wounds probably can serve as vectors under some conditions, but they would seem to be of secondary importance. These other insects would include species of the predaceous families Staphylinidae, Colydiidae, Ostomidae (Tenebrionidae), and Cucujidae; Histeridae of the order Coleoptera; and possibly some Diptera.

A NEW TECHNIQUE FOR DEMONSTRATING THE PRESENCE OF THE OAK WILT PATHOGEN ON THE BODIES OF INSECTS.—During the early phases of oak wilt investigations at West Virginia University, attempts were made to demonstrate the presence of spores of the oak wilt fungus on insects (mainly species of Scolytidae and Nitidulidae) collected from the infected trees. Many techniques were employed in efforts to recover the fungus from the insects. These included allowing beetles to run over various types of agar in petri dishes, washing beetles and streaking the washings on various types of agar, and placing live beetles (mainly species of Scolytidae) in small oak wood cups placed on agar. Although the fungus has been cultured from the bodies of nitidulids caught in nature (39), none of the various methods used was satisfactory. The greatest difficulty encountered was the rapid growth of various contaminants, which quickly covered the test plates. Because of these difficulties other methods were sought.

Leach et al (30) had reported the ability of nitidulid beetles to act as agents of spermatization by carrying spores from a mycelial mat of one compatibility type to a mat of the opposite type. This observation led to the development of a technique that involved use of petri-dish cultures of both compatibility types of the oak wilt fungus for detection of the presence of spores of either compatibility type carried by insects.

Seven- to 10-day cultures of the pathogen grown in petri dishes on maltose-Casamino Acids agar were used (1). Younger or older cultures than this often do not produce fertile perithecia. All cultures were incubated at either 20° or 25°C. Three or 4 colonies of a single compatibility type were grown in each petri dish.

In preliminary tests, nitidulid beetles were collected and placed on either A or B compatibility-type cultures, allowed to feed for 24 hours, and then transferred to cultures of the opposite compatibility type, 1 beetle per test culture.

Three days later the beetles were removed and the cultures examined for the presence of perithecia. Positive results were obtained in most cases. Of 20 insects representing 4 species of nitidulids, 16 gave positive results.

Although some contaminants were present in the spermatized cultures, easily recognized fertile perithecia were nevertheless produced.

In an attempt to determine whether the spermatizing technique could be used to demonstrate spores on or in insects found in association with mycelial mats of the oak wilt fungus, several species of Nitidulidae, 1 of Histeridae, 1 of Colydiidae, and 1 of Ostomidae were collected from mycelial mats on infected trees. One mat was producing fertile perithecia when the insects were collected; the others had no perithecia when examined on the tree but did form them when taken to the laboratory.

Eleven of these insects were placed separately in tubes containing a small amount of sterile distilled water. The tubes were shaken intermittently by hand, and at the end of 3 hours the insects were removed. One drop from each tube was placed, by means of a sterilized pipette, on type A colonies of the oak wilt fungus and another on type B colonies in a separate petri dish. The drops were spread over the colonies with a flamed loop. Either 1 or 2 plate cultures, each

consisting of 3 or 4 colonies, of each type were used in each test. Other cultures of both A and B types were treated with distilled water as checks.

The treated plates were examined for perithecia after incubation at 20°C for 3 days. Fertile perithecia were found on plates spermatized with washings from 8 of the 11 individual insects (Table 3). As only A-type cultures produced fertile perithecia when spermatized with the washings, it must be concluded that only B-type conidia were present on the insects.

Eleven other insects collected from the mats were placed individually in sterile dry test tubes at the time of collection; 2 individuals were *G. sanguinolentus*, 3 were *G. fasciatus*, 3 were *G. confluentus*, 1 was of the genus *Epuraea*, and 2 were *Platystoma* sp. (Histeridae). These were tested for the presence of viable spores of the oak wilt fungus by later randomly placing the insects on 7-day-old cultures of *E. fagacearum* of either type A or type B, 1 beetle per plate. After 3 days, the test cultures were examined. Fertile perithecia were produced on test cultures of at least 1 of each species. All 3 of the specimens of *G. fasciatus* tested produced perithecia. This insect and the specimens of *Platystoma* sp. tested brought about perithecial formation on test cultures of both compatibility types, indicating that both types of spores were carried by different individuals collected at the same location.

When the insects were collected, samples were taken from 7 selected mats on which there were no insects and over which there were no open bark cracks. The mat samples consisted of small strips of mycelium cut from the mats with a sterilized scalpel. Each strip, 1 from each mat, was placed in separate tubes containing sterile distilled water. The strips were kept in the tubes for about 3 hours. The tubes were shaken occasionally during a 3-hour period, after which the strips were removed from the tubes, drops of each suspension were placed on separate colonies of A and B types and spread over the colonies with a sterilized loop. Two plates of each compatibility type were used for each suspension. After incubation at 20°C for 3 days, all A-type cultures had fertile perithecia, whereas there were none on the B-type cultures.

TABLE 3.—*Production of fertile perithecia by colonies of compatibility type A and B of E. fagacearum to which was added wash-water from single insects collected from fungus mats on infected oak trees*

Species of insect washed	Tested on[a] Type A	Type B
Glischrochilus sanguinolentus	P,0	0,0
	0	0
	0	0
G. fasciatus	P	0
	0	0
G. confluentus	P,P	0,0
Colopterus morio	P	0
Epuraea sp.	P	0
Platystoma sp. (Histeridae)	P	0,0
Tenebroides sp. (Ostomidae)	P,0	
Laemophloeus sp. (Colydiidae)	P	0

[a] P, fertile perithecia produced on 1 test culture; 0, no fertile perithecia produced.

The presence of fertile perithecia on one of the mats from which insects were collected indicates that the insects had brought A-type spores to this mat, since the unexposed mats were shown to be a compatibility type B. Thus, insects collected from the exposed mats could have been carrying spores of both compatibility types. This may have been the case with the insects that spermatized both types in the tests where insects were fed on the test cultures.

The insects used in these experiments had been in close association with the oak wilt fungus and therefore may have been rather heavily contaminated with the spores. The results indicate, however, that the technique could be applied with a reasonable degree of success to insects contaminated with spores by normal contact with oak wilt mats.

TEST OF INHIBITION OF E. FAGACEARUM BY ASSOCIATED FUNGI.—*Graphium rigidum* and *Ophiostoma pluriannulata*, the fungi consistently found associated with nitidulids in nature, and an undetermined species of *Endoconidiophora* were often found growing on agar plates on which washings of insects had been streaked or on which insects had been placed. Usually, when any of these fungi were found, it was difficult to determine the presence of the oak wilt fungus. Tests were made to determine whether the presence of these fungi inhibited germination of the oak wilt fungus spores or simply overgrew the oak wilt organism.

Separate conidial suspensions of comparable density were prepared in equal volumes of distilled water from cultures of *G. rigidum*, of *O. pluriannulata*, of *Endoconidiophora* sp., and of each compatibility type of *E. fagacearum*. These spore supensions were then mixed so that each of the saprophytic fungi was combined with spores of the oak wilt fungus of both compatibility types. Sterilized pipettes were used to transfer drops of each mixed spore suspension to 2 separate colonies of *E. fagacearum* of the compatibility type opposite to that of the colony used in making up the spore suspension. The drops of each suspension were spread over the colonies with a flamed loop.

Perithecia formed on 12 of the 16 colonies so spermatized. The results of these limited experiments suggest that the presence of these saprophytic fungi exerts no pronounced effect on the normal functioning of the conidia in sexual reproduction.

ISOLATION OF THE OAK WILT PATHOGEN FROM BODY AND FECES OF NITIDULIDAE.—Following the first 2 reports on the experimental insect transmission of oak wilt by Nitidulidae in the summer of 1953 (13, 34), work was started to determine whether the spores of the oak wilt fungus could pass, in a viable condition, through the intestinal tract of nitidulids, as has been demonstrated with other spores and insect vectors (29). It had been assumed that insect transmission of the oak wilt fungus by nitidulids is purely mechanical in nature (34). Bart and Griswold (5) dem-

onstrated the viability of the conidia of the oak wilt fungus in the feces of *Drosophila melanogaster*. During the early stages of the present work, microscopic examination revealed conidia of the oak wilt fungus in much of the fecal material of nitidulids that had fed on cultures of the fungus (Fig. 17).

Various techniques were used in attempts to isolate the oak wilt fungus from fecal pellets. When pellets were plated on several types of agar, including water agar, the oak wilt fungus was quickly overgrown by yeasts and bacteria. The possibility of using a modified spermatizing technique, in which fecal pellets were used for spermatizing both A- and B-type cultures, was tested.

Two species of nitidulid beetles, *G. fasciatus* and *G. sanguinolentus*, were fed on A-type cultures supplied fresh every other day for 1 week. The beetles were then removed from the feeding dishes by means of flamed forceps, glued upside down on glass slides previously washed in alcohol, and then brushed several times with 95 per cent alcohol to eliminate surface contamination. Defecation was observed under a stereoscopic microscope. At the time of defecation, the pellets were collected with a flamed needle. After a small drop of sterile distilled water had been placed upon the mycelium of a B-type test culture, a test pellet was added to the water droplet. Pellets were also used on A-type cultures as checks. Under a stereoscopic microscope each pellet was crushed and dispersed in its drop of water and spread over the colony with a flamed needle. These cultures were incubated for 3 days at 20°C and then examined for the presence of fertile perithecia.

Three separate series of experiments were made. A total of 75 colonies were spermatized with the feces collected from 45 nitidulids previously fed on A-type cultures; 66 of the test colonies were B type and 9 were A type. Forty-six of the 66 B-type test colonies produced fertile perithecia. The number of fertile perithecia produced on each colony varied from 2 to 30, most colonies producing from 15 to 20. None of the A-type test colonies produced fertile perithecia when spermatized with fecal pellets from beetles that had fed on A-type cultures.

Similar experiments were later carried out with other species of nitidulids. The feeding of the beetles varied in that some were fed on cultures that were producing fertile perithecia. Such beetles were assumed to have fed on both compatibility types. In most cases, however, the beetles were fed on cultures of only 1 compatibility type prior to collection of the fecal pellets.

Two additional species of nitidulids, *G. confluentus* and *C. lugubris*, were found to pass viable spores through their intestinal tracts. Of 23 pellets from 13 individuals of *G. confluentus*, 7 pellets from 5 individuals contained viable spores. One of these had fed on perithecia-bearing cultures, and 2 pellets from it contained viable spores. One pellet collected from this insect was crushed on a slide and observed under the microscope; ascospores were present (Fig. 18). Two individuals of the species *G. sanguinolentus* produced 3 pellets, all of which contained viable spores. A single individual of *C. lugubris* was used, and the 1 pellet tested contained viable spores.

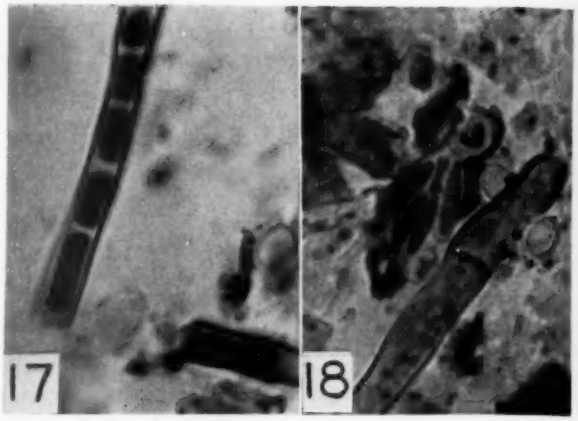

FIG. 17–18.. Photomicrographs of spores found in freshly excreted fecal pellets of nitidulids that had fed on cultures of *E. fagacearum*.—Fig. 17. Conidia.—Fig. 18. Ascospores. Approximately 3000×.

The consistently positive results obtained in these experiments show that the spermatizing technique utilized is efficient in revealing the presence of viable spores of the oak wilt fungus on or within the bodies of insect vectors. The technique may be useful in determining the presence of such spores in other material. It also may be possible to extend its use to other heterothallic fungi.

THE SUCCESSFUL INOCULATION OF TREES WITH FECAL PELLET MATERIAL.—A slightly different technique for collecting the fecal pellets was used. Previous trials had shown that washing contaminated beetles in several changes of distilled water removed most of the surface contamination. After feeding, each insect was placed in a separate petri dish containing a piece of filter paper. Fecal pellets were collected with a flamed needle as they were dropped, and placed in 0.5 ml of sterile distilled water in small fermentation tubes. Twelve hours later they were crushed thoroughly, and sterile distilled water was then added to each tube to bring the volume to 1 ml. Fecal pellets were collected from *Cryptarcha ample, G. fasciatus, G. sanguinolentus, G. quadrisignatus, and C. hemipterus*. Pellets from *C. ample* were used to make up 2 suspensions. Fresh wounds were made in trees of the red oak group by making a chisel cut into the sapwood. Measured amounts of each suspension were injected into each wound by means of hypodermic syringes. When practical, 2 trees were inoculated with a single suspension, and approximately the same amount of the inoculum was used in each case. The check trees received 0.25 ml of a spore suspension made from cultures of *E. fagacearum* of known compatibility type. Fifteen trees were inoculated with 6 fecal-pellet suspensions, and 6

check trees were inoculated with a spore suspension. A check tree was used with each group of trees inoculated with the pellet suspension from each of the 5 species.

On July 30, 4 of the check trees and 1 of the trees inoculated with 0.5 ml of the pellet suspension from *G. fasciatus* showed typical oak wilt symptoms. The oak wilt pathogen was recovered from all the trees sampled by the procedure previously described. On August 3, an additional tree that had been inoculated with 0.2 ml of the pellet suspension collected from *C. ample* showed symptoms and was confirmed by laboratory culture. No other trees developed symptoms by the time of the first killing frost.

Although only 2 of the 15 trees inoculated with the pellet suspensions developed oak wilt, as compared to 4 of the 6 inoculated with spore suspensions, the results show that fecal pellets can, under suitable conditions, serve as effective inoculum.

Discussion.—Since the subcortical mycelial mats have been shown to be sources of abundant inoculum and fresh wounds have been demonstrated to serve as infection courts for the oak wilt fungus, it is logical to consider insects found most frequently in both situations the important vectors. Many species of insects occur in both situations. Of these, species of the family Nitidulidae (Coleoptera) are the most numerous. The insect transmission experiments of 1953 and 1954 reported here, and work done elsewhere (23, 32, 34, 36), have demonstrated conclusively that nitidulids are vectors of oak wilt. The results of the transmission experiments, the constant association of nitidulids with a source of abundant inoculum on which these insects feed in nature, the presence of nitidulids in large numbers on proved infection courts (fresh wounds), and the demonstration that those nitidulids that have been associated with the mycelial mats are usually contaminated inside and out with viable spores of the pathogen fulfill the requirements for the rules of proof of insect transmission set forth by Leach (27). Although the positive results reported here were relatively few, the transmission experiments show that certain species of Nitidulidae can transmit oak wilt. Three species, *G. sanguinolentus*, *C. semitectus*, and *C. lugubris* (Fig. 13, 14, 15), were shown to be capable of transmitting the disease when each was used alone on a tree. Another nitidulid, *G. confluentus*, was not shown to transmit the disease when used alone, but transmitted it in 1 case when in combination with *C. lugubris* in 1953. In these experiments, insects contaminated either with conidia and ascospores or with conidia alone transmitted the disease effectively.

From other reported work, it is evident that under suitable conditions other insects may transmit oak wilt. Griswold (16) reported transmission by the pomace fly, *Drosophila melanogaster* Meig., to a single oak seedling in greenhouse experiments. Later Griswold and Bart (17) reported transmission to a seedling in the greenhouse by the scolytid, *Pseudopityophthorus pruinosus* (Eichh.). It is also possible that some of the predaceous insects (mainly Coleoptera) found on mats and wounds may transmit oak wilt under some conditions, as indicated by the work of McMullen et al (32), but they are here considered to be of minor importance as their presence follows but does not precede the infestation of fresh wounds and mats by nitidulids. Although species of Scolytidae occasionally were found on mats and wounds, no evidence was obtained to indicate that they are important in spreading oak wilt. Although these beetles are found in wilt-killed trees, they are rarely found in association with mycelial mats. The common ambrosia beetles rarely attack healthy trees, but they may bore into the wood of dying oak-wilt trees and emerge sometime later through the wood of the diseased tree. In doing so, it is possible that these beetles would become contaminated with inoculum, but evidence of this was not obtained.[3]

From the available evidence (10, 11, 24), most natural overland spread occurs during the spring, and most of the successful insect inoculations, here and elsewhere, were made during May or early June (23, 32, 34, 36). Wounds made in the spring are infection courts and are more attractive to nitidulids than similar wounds made at other times of the year. Many of the same insect species present on wounds during the spring were also found on mats at the same time. The mats found in the fall were infested with nitidulids, but wounds made in the fall were not attractive to these insects. Curl (11) reported that mats provided the greatest potential inoculum for oak wilt spread during March, April, and May in Illinois. The importance of spring mat formation is further emphasized by the reports of Jeffrey (24) and Craighead et al (10) that natural infection of oaks occurs most frequently during the spring. Work by Boyce (6) indicates that some of the trees killed by oak wilt and felled the same summer may produce mats in the fall. These mats were found heavily infested with insects; however, the fall-formed mats were badly disintegrated by the following spring. This would indicate that mats formed in the fall may not be as important sources of inoculum as those formed in the spring. It should not be concluded, however, that no spores survive the winter on mats or in or on the bodies of insects. Laboratory experiments on the longevity of spores under various conditions of relative humidity and temperature have shown that both conidia and ascospores are long-lived under low-temperature conditions (15, 25, 31). If insects feeding on mats in the fall are able to carry viable spores of the oak wilt fungus over winter, either externally or internally (39), then fall-formed mats might serve

[3] Since this paper was submitted for publication, Stambaugh et al have reported such evidence. (Stambaugh, C. L., C. L. Fergus, F. C. Craighead, and H. E. Thompson. 1955. Viable spores of Endoconidiophora fagacearum from bark and wood-boring beetles. U. S. Dept. Agr. Pl. Dis. Rptr. 39: 867-871.)

as possible sources of spring inoculum. The length of time spores of the oak wilt fungus are retained internally by nitidulids is not known; however, if they are retained during hibernation or quiescent periods, the spores would be in a protected environment and might survive longer than those adhering to the outside of the insect's body.

The constant association of species of Nitidulidae with *G. rigidum* and *O. pluriannulata* is very probably a symbiotic relationship of long standing. The fungi are obviously benefited by being disseminated to suitable substrates by the insects, which in turn receive nutritional benefit from the fungi. Fresh wounds serve as a suitable environment for the insects and apparently provide all the required factors for growth and sporulation of the 2 fungi. The oak wilt fungus is probably a new member of this symbiotic relationship. The pressure cushions of the oak wilt fungus open wounds through the bark to the cambium. Nitidulid beetles are attracted to these wounds at least partly by the odor of the oak wilt fungus. Here the nitidulids find an environment and a food supply similar to that provided by wounds that occur in healthy trees, where they are usually associated with *G. rigidum* and *O. pluriannulata*.

The oak wilt fungus can not live long as a saprophyte, as can *G. rigidum* and *O. pluriannulata;* thus, it must have living trees in which to survive and propagate. Apparently the fungus requires suitable wounds as infection courts and such wounds may be found only on widely separated trees during the limited season most favorable for infection, and there is some evidence (40) that the age of wounds is also a limiting factor in infection. Spores of *E. fagacearum* are not adapted to wind dissemination, but probably can be spread by insects for long distances. Without a vector, the oak wilt fungus in its original location could spread and maintain itself only through root grafts. The scattered occurrence of oak wilt and its absence in certain areas where both nitidulids and oaks are abundant indicate that the association of the nitidulids with the disease is of relatively recent origin. If this is true, the extent of the association can be expected to increase from year to year, with a corresponding increase in the rate of overland spread. Thus, control of the disease in regions where the oak wilt fungus and the nitidulids are associated only in localized areas would seem to be amply justified.

Although the nitidulids are effective vectors of oak wilt, they appear to be relatively inefficient when compared to some vectors of other diseases. Wounds seem to be necessary infection courts for overland spread, and the nitidulids cannot make the wounds that they infest. Moreover, their efficiency appears to be limited by the necessity for fresh wounds made during a limited period in the spring of the year. These wounds probably can be of many types, but those most attractive to the nitidulid insects are moist wounds with bark at least partially separated from the sapwood.

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THE RED CLOVER VEIN-MOSAIC VIRUS IN WISCONSIN[1]

Clinton H. Graves, Jr., and D. J. Hagedorn

SUMMARY

Red clover, sweet clover, alsike clover, Ladino clover, and black medic have been found to harbor the red clover vein-mosaic virus in nature. Symptom expression of this virus was found to be characteristic and distinct from that of other viruses. In general the symptoms consisted exclusively of a vein chlorosis.

Incidence studies made in Wisconsin during 1951 and 1952 revealed a striking prevalence of red clover vein-mosaic virus among most of the named hosts. The virus was endemic among leguminous weeds along roadsides and in waste places, suggesting that such plants are important in perpetuation of the virus.

The virus was found in leaves and stems, in crowns, and in roots of red clover and sweet clover.

Indirect evidence of overwintering of the virus in red clover, sweet clover, and alsike clover plants was obtained.

The recent discovery by Hagedorn and Hanson[2] of the identity of Wisconsin pea stunt virus[3] with red clover vein-mosaic virus[4] helps explain the yearly encroachment of an important disease of peas (*Pisum sativum* L.). Numerous other virus types have been found also among perennial legumes in nature; the array of symptoms produced in clovers by these viruses, alone and in various combinations, presents a rather bewildering picture. Red clover vein-mosaic virus, however, has been found to be quite distinct in symptomatology, in contrast to most legume viruses.

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[2] Hagedorn, D. J., and E. W. Hanson. 1951. A comparative study of the viruses causing Wisconsin pea stunt and red clover vein-mosaic. Phytopathology 41: 813-819.

[3] Hagedorn, D. J., and J. C. Walker. 1949. Wisconsin pea stunt, a newly described disease. Jour. Agr. Res. 78: 617-626.

[4] Osborn, H. T. 1937. Vein-mosaic virus of red clover. Phytopathology 27: 1051-1058.

The present study was undertaken to 1) determine the most common natural leguminous hosts of red clover vein-mosaic virus; 2) to study the range in symptom expression among natural leguminous hosts; and 3) to investigate the incidence and seasonal aspects of red clover vein-mosaic virus among natural leguminous hosts, in comparison with the complex of other viruses found among these hosts. A preliminary report was made earlier.[5]

NATURAL LEGUMINOUS HOSTS OF RED CLOVER VEIN-MOSAIC VIRUS.—Over 300 leguminous plants suspected of harboring red clover vein-mosaic virus were collected from 5 Wisconsin pea-growing counties and transplanted into clay pots. After close observation for 1–3 weeks, the plants were grouped according to symptom expression. Excessive repetition was avoided by taking a number of plants at random from each symptom type for further study and tests. Extracts from these plants then were used to inoculate Wisconsin Perfection and Perfected Wales peas, red clover (*Trifolium pratense* L.), and Idaho Refugee beans (*Phaseolus vulgaris* L.). Comparisons of symptoms on these plants were made with those produced by the original virus incitant of Wisconsin pea stunt.

Typical red clover vein-mosaic response was obtained with extracts from naturally infected red clover, sweet colver (*Melilotus officinalis* Lam. and *M. alba* Desv.), alsike clover (*Trifolium hybridum* L.), Ladino clover (*Trifolium repens latum* McC.), and black medic (*Medicago lupulina* L.). Although white clover (*Trifolium repens* L.), alfalfa (*Medicago sativa* L.), and vetch (*Vicia angustifolia* L. Reichard) populations were examined widely, no indicative symptoms were found, and all such plants tested were free of this virus.

SYMPTOMATOLOGY. — Symptom expression of the natural hosts to infection by red clover vein-mosaic virus was distinctive, consisting almost exclusively of a vein chlorosis. The range of symptoms found in red clover varied from a severe chlorosis and yellowing of the main veins only (Fig. 1, C) to a lighter chlorosis of the fine veinlets only (Fig. 1, D, E). The most typical and common expression (Fig. 1, B) was a definite chlorosis of both the main and secondary veins. In some cases in nature, the presence of a second virus influenced symptom expression (Fig. 1, F, G). In such cases, however, the vein chlorosis symptom was usually distinct in appearance and easily detected.

The symptom shown in Figure 1, H is considered the earliest one following the feeding of an infective insect. This type of symptom occurs usually on only 1 leaf of a plant, commonly involves the veins and veinlets of only a small portion of the leaf, and is of widespread occurrence in new seedings in the late fall. When such plants are potted and kept in a greenhouse under optimum conditions, typical vein-mosaic symptoms will eventually appear on all parts of the plant. The segmented reaction may result from high virus concentration in localized areas following the feeding of a viruliferous insect.

Symptoms of infection of other legumes by red clover vein-mosaic virus were similar to those in red clover (Fig. 1, I, J, K). The segmented type of reaction (Fig. 1, L) was found occasionally in Ladino clover.

Typical red clover vein-mosaic virus was isolated from only 2 plants of black medic. Symptoms consisted of very slight vein-clearing and a general light yellow mottle.

INCIDENCE.—Virus incidence studies among the natural hosts of red clover vein-mosaic virus were made in Dodge, Ozaukee, Marathon, Chippewa, and Barron counties of Wisconsin during 1951 and 1952. Red clover vein-mosaic was distinguished readily from other virus diseases on the basis of symptoms. The other virus diseases could not be distinguished from each other by symptoms alone, hence they were recorded under a single category. A number of townships in which canning peas were grown were selected at random within each county or area under consideration, and within each township a number of field and roadside samples were taken. In most cases the average number of red clover fields studied per county was slightly over 10, and the number of roadside samples averaged about 20.

Actual sample counts were taken of diseased and healthy plants within a given area. Intrafield samples were taken by use of square rod quadrats, the number taken per field being dependent upon field size and clover stand. For roadside determinations clover patches along the roadsides were taken at random to avoid personal bias. A definite area was marked, the size depending upon the intensity of the stand, and all diseased and healthy plants were counted.

At intervals, the accuracy of the counts was checked by selecting at random plants considered infected with red clover vein-mosaic virus and testing them for this virus. The percentages of positive results obtained from such plants were almost as high as that obtained from check plants inoculated with a known culture of the virus.

Surveys covering essentially matched samples were made at different periods during the season to check for seasonal variations. All results have been analyzed statistically and are available.[6]

Incidence of red clover vein-mosaic virus was higher than that of other viruses in almost all cases (Table 1). The difference was significant statistically. Within red clover fields there was ordinarily a gradual increase in incidence of virus diseases as the season progressed. Although individual county averages for red clover vein-mosaic incidence over the 2-year peri-

[5] Graves, C. H., Jr. 1953. Incidence of the red clover vein-mosaic virus in Wisconsin clovers. (Abs.) Phytopathology 43: 473.

[6] Graves, C. H., Jr. 1954. The red clover vein-mosaic virus, its natural leguminous hosts, symptomatology, incidence, and transmission phenomena in Wisconsin. Ph.D. thesis. University of Wisconsin. 116 p.

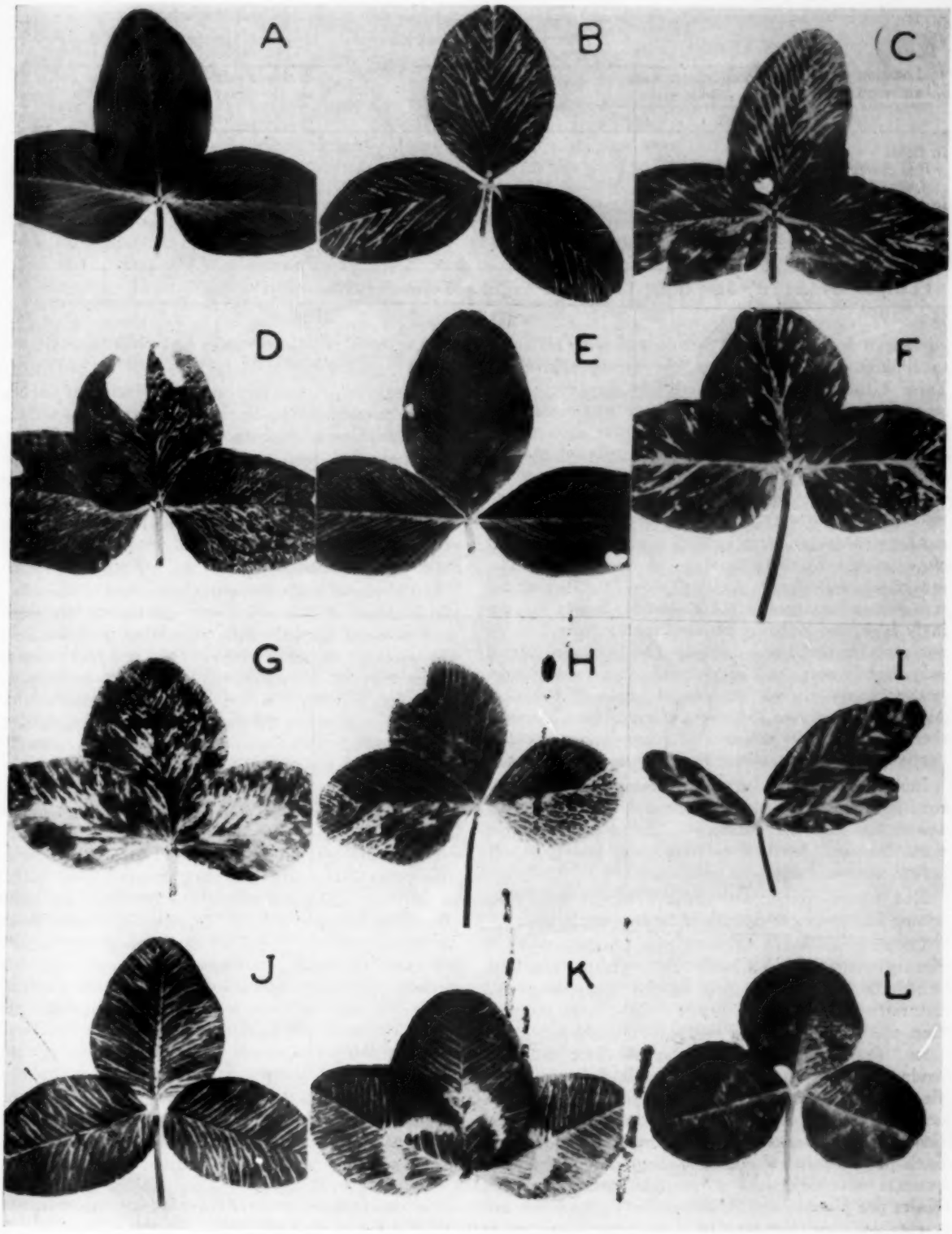

FIG. 1.—Variations of red clover vein-mosaic virus symptom expression in naturally infected forage legumes. A) Healthy red clover leaf. B-H) Variations in diseased red clover: B represents the most common type expression; F and G show evidence of a virus mixture. I) Diseased sweet clover; J) Diseased alsike clover; K, L) Diseased Ladino clover.

TABLE 1.—*Incidence of virus diseases among leguminous plants within important pea-growing sections of Wisconsin at 2 periods during 2 growing seasons*

Location and crop	Percentage of plants infected by indicated viruses							
	1951[a]				1952[a]			
	Red clover vein-mosaic virus		Other viruses		Red clover vein-mosaic virus		Other viruses	
	Early	Late	Early	Late	Early	Late	Early	Late
In fields								
Red clover	1.5	10.7	3.1	1.7	6.9	12.4	1.8	1.9
In waste areas								
Red clover	6.6	28.4	7.8	5.8	11.3	10.0	2.8	4.3
Alsike clover	14.5		5.0		10.5	4.2	1.0	1.2
Sweet clover					13.9	3.8	3.8	3.0

[a] Early = May and early June study; Late = Late August and early September study.

od ranged from less than 1 per cent in some instances to slightly over 25 per cent, the averages given in Table 1 for all 5 counties were less divergent. The seasonal increase during both seasons was highly significant statistically in most cases. The variation among counties of different latitude was significant statistically, incidence being significantly lower in the northernmost counties. Other viruses did not show a seasonal increase or a variation in respect to latitude.

Along roadsides, virus incidence was greatest in red clover stands, with that in alsike clover and sweet clover stands following closely (Table 1). Red clover vein-mosaic incidence in red clover stands was surprisingly high, the 5-county means ranging from 7 to 28 per cent for the 2-year period. The incidence varied only slightly over the 2-year period in any of the counties, with no distinct pattern of seasonal behavior. Among alsike clover and sweet clover plants, however, there was a definite seasonal decrease each year. The decline apparently was due to defoliation by insects.

Incidence of other viruses among the red clover roadside stands varied from about 1 to 15 per cent among the counties considered and averaged 5 per cent. Seasonal fluctuations were rare, and those observed were variable.

Red clover vein-mosaic incidence was negligible among Ladino clover and black medic populations.

LOCATION OF VIRUS IN PERENNIAL HOSTS.—Severely diseased red clover and sweet clover plants, from both artificially inoculated stock in the greenhouse and naturally infected material in the field, were removed from the soil and divided into several portions. A different sterilized razor blade was used for each cut made from the top of the plant to the lower rootlets. Each portion of the plants (lower rootlets, crown, leaf, and stem) was then handled individually in a manner that prevented contamination of one with the other. Each plant section was chopped and ground, and the extracts were then used to inoculate young Perfected Wales pea plants.

Following the usual incubation period, typical red clover vein-mosaic symptoms became evident on all inoculated test plants, an indication that all such portions of both red clover and sweet clover contain the virus.

DISCUSSION.—These host and virus-incidence studies provide valuable evidence of the importance of certain perennial legumes as source plants for viruses of other economic crops such as peas and beans. Red clover vein-mosaic occurs in strikingly high incidence and is generally prevalent over all areas considered. Other virus diseases, though not present in such high percentage, are endemic throughout clover populations, both cultivated and volunteer.

In red clover fields, there was a gradual introduction and build-up of both red clover vein-mosaic and other virus diseases from the time of seeding until the field was diverted to other crops. This provides definite evidence of the potentialities of this plant as an overwintering host for the red clover vein-mosaic virus. Among plants along the roadsides and waste areas, a rather static incidence was evident, apparently governed and maintained by the natural processes of reinfection and dying out.

The association of these natural hosts with the habits of the known vector of this virus resolves the yearly cycle by which this virus reappears in pea fields year after year. Peas are the most favored food plants of the most important vector, the common pea aphid (*Macrosiphum pisi* Kelb.). Alfalfa, red clover, alsike clover, sweetclover, and other clovers, however, usually serve as perennial reservoirs for the aphids during periods when peas are not growing. In the northern states, the pea aphids overwinter as eggs on stems and fallen leaves of alfalfa and clovers. The aphids feed on these hosts for several generations before winged forms migrate to the pea fields.

Transmission of the red clover vein-mosaic virus by a small clover aphid, *Myzocallis ononidis* Kalt.,[7] also might help to explain virus movement and the high virus incidence in clover populations.

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[7] See footnote 6.

POSTHARVEST TREATMENTS FOR REDUCTION OF BROWN AND RHIZOPUS ROTS OF PEACHES [1]

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SUMMARY

During a 4-year period, 67 chemicals (55 as sprays or dusts and 12 as fumigants) were tested as postharvest treatments for reduction of decay of peaches inoculated with spores of *Monilinia fructicola* (Wint.) Honey or of *Rhizopus stolonifer* (Fr.) Lind. Because of the rapidity of symptom development, the effectiveness of a chemical against Rhizopus rot could be determined in 3 days at 75°F, whereas 6 days was necessary to evaluate it against brown rot. Final evaluation of a chemical against both decays was made after 6 days, the time necessary for a majority of the fruit to become fully ripe.

Several chemicals were effective in reducing the 2 types of decay. Sprays of Orthocide 50 Wettable and Isothan Q15 were equal to or more effective than sulfur dust in reducing brown rot; but like sulfur dust, they were ineffective against Rhizopus rot. When used as fumigants and volatilized either with or without heat, both tetrachloroethylene and trichloroethylene effectively reduced Rhizopus rot but were ineffective against brown rot. Dowicide A spray and a combination treatment of sulfur dust followed by fumigation with tetrachloroethylene effectively reduced both brown rot and Rhizopus rot.

The materials reported in this paper were tested for fungicidal properties only, and it is not known whether they would leave residues toxic to man when used as indicated.

For years it has been customary to cool peaches as rapidly as possible and to ship them under standard refrigeration to delay development of decay. The cooled fruit arrives at destination in a hard-to-firm condition and often without any visible decay even though the spores of decay-producing fungi may be present on the surface. Precooling usually does not reduce the spore load. Consequently the spores may germinate, infect the fruit, and cause decay during the 4–6 (or even more) days at room temperature usually required for the fruit to ripen after arrival at market. A fungicidal treatment that kills the spores or inhibits their germination is therefore necessary if the number of peaches that ripen without decay is to be increased.

Numerous workers (1, 3, 4, 5, 6, 7, 8, 9) have reported the effects of fungicidal treatments on brown rot of peaches, but fewer (4, 5, 7, 8, 9) have reported their effects on Rhizopus rot. Treatments to reduce both decays were tested during a 4-year period, 1951-54. Results are presented for only spray or dust materials that effectively reduced at least 1 of these decays and for certain fumigants that previously had been reported (9) to be very effective against both.

MATERIALS AND METHODS. — Commercially picked peaches were obtained in 1951 from packing sheds in Georgia and West Virginia and in 1952, 1953, and 1954 from wholesale houses in Washington, D. C. Before the start of an experiment, the over-ripe, the bruised, and the otherwise injured fruit was removed, and the hard-to-firm peaches remaining were composited into experimental samples.

To give consistently high infection, the fruit was inoculated with spores of either the brown rot fungus (*Monilinia fructicola* (Wint.) Honey) or the Rhizopus rot fungus (*Rhizopus stolonifer* (Fr.) Lind.), usually 2 or 3 hours before the test fungicides were applied. Spores were obtained from peaches infected with the respective fungi, and the inoculum consisted of a spore suspension in beef-peptone broth to which 0.1 per cent Tween 20 (polyoxyethelene sorbitan monolaurate) was added. The concentration was 30–40 Monilinia spores or 70–80 Rhizopus spores per low-power field of a microscope. Uninjured peaches were inoculated with Monilinia. Consistently high infection was obtained by spraying the spore suspension on uninjured fruit as they revolved on a roller table. In contrast, injury was necessary for consistent infection with Rhizopus. In 1951, 3 shallow pin-pricks were made on each cheek, and then the peaches were inoculated by spraying them with the spore suspension as they revolved on a roller table. In the other years the injury was a shallow cut, resembling a finger nail cut, on each cheek, and the fruit was inoculated by dipping the cut surface into the spore suspension.

In an attempt to obtain complete coverage, sprays were applied by atomizers as the peaches revolved slowly on a roller table. For the dust treatments, the roller table was covered with a hood, the material was dusted from an atomizer flask for 15 seconds, and then the dust was allowed to settle on the revolving peaches for 1 minute. In most cases a very heavy and complete dust coverage was obtained.

Fumigation was done in 10-cu.-ft. metal chambers, each equipped with a small electric hot plate and fan. The experimental samples in open ½-bushel baskets were placed in the chambers, and the liquid fumigant, in shallow receptacles, was put on hot plates set for low heat. The chambers were then closed with a water seal. One method of fumigant volatilization was to use the hot plates for 5 minutes, followed by fan circulation of the vapors for 5 minutes. The other

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method was volatilization of the fumigant at room temperature (75°–85°F) while the fan circulated the vapors for 5 minutes. Operation of the hot plates and fans increased the temperature of the air in the chambers about 10° and that of the fruit about 3°. Both returned to room temperature in about 3 hours. All fumigation treatments were conducted within 2 or 3 hours after inoculation and lasted for 24 hours. Carbon dioxide accumulated in the chambers during this period, reaching a concentration of 7-8 per cent. The concentration was essentially the same in the control and treatment chambers, however, indicating that the treatments had not appreciably affected the respiratory activity of the fruit.

In each test year, a fungicidal treatment consisted of 4 replicates of 3 lots of approximately 50 peaches. One lot remained noninoculated, the second was inoculated with spores of Monilinia, and the third was inoculated with spores of Rhizopus. Replication consisted of conducting each experiment on 4 dates, each with peaches of a different variety or from a different source. Since infection of noninoculated fruit was usually too low for evaluation of the effectiveness of a fungicide in reducing decay, the data presented show only the effects of treatments on peaches inoculated with Monilinia or Rhizopus.

After treatment, the peaches were individually wrapped in copper-impregnated paper (Hartman wraps) to reduce the spread of decay from peach to peach within the basket and were held at a constant temperature of 75°F for 6 days. As an additional precaution against the spread of decay, the peaches were inspected after 3 days and all decayed fruit was recorded and removed.

During the first 3 days at 75°, brown rot averaged 13 per cent more on untreated inoculated peaches than on noninoculated ones. After 6 days, the decay increase averaged 56 per cent. This indicates that a holding period of 3 days is not sufficient for development of brown rot and for evaluating the effectiveness of a fungicide against the brown rot fungus. The comparable values for Rhizopus rot were 73 and 77, respectively, indicating that 3 days at 75° is usually sufficient for development of Rhizopus rot. Because of the difference in rapidity of decay development, both the 3- and the cumulative 6-day decay readings are presented.

A fungicide was not used after an initial test unless it caused at least a 50 percent reduction in brown or Rhizopus rot. In the case of brown rot, tests with a new material were not continued unless its effectiveness was equal to or better than that of the standard commercial treatment, sulfur dust. This dust, however, was almost completely ineffective against Rhizopus rot.

Regardless of how effectively a material reduces decay it is not satisfactory if it causes injury or off-flavor to the fruit or leaves an unattractive residue or one toxic to man.

Results.—Many of the 55 chemicals tested as sprays or dusts over a 4-year period gave good to excellent reduction of either brown rot or Rhizopus rot, but most of them failed in at least 1 of the criteria mentioned in the preceding section. Others were not sufficiently tested to be included in this report. Detailed reports on the 1951 and 1952 studies were multilithed (4, 7). Results obtained with the 4 materials most effective in reducing 1 or both types of decay during 4 years of testing are given in Table 1.

The 3 newer materials reduced brown rot during the first 3 days at 75°F by a higher percentage than did sulfur dust. Reduction of this decay by Dowicide A (97 per cent sodium *o*-phenylphenoxide) was outstanding, and it alone of the 4 materials also reduced Rhizopus rot by more than the required 50 per cent. In 6 days at 75°, Orthocide 50 Wettable (50 per cent captan or *N*-(trichloromethylthio)-4-cylohexene-1,2-dicarboximide) reduced brown rot somewhat more than sulfur dust, and reduction by Dowicide A and Isothan Q15 (20 per cent 2-dodecylisoquinolinum bromide) equaled that by sulfur dust. At that time, Dowicide A was the only material that had reduced Rhizopus rot by 50 per cent. Isothan Q15, on the contrary, apparently had caused a rather large increase of this

Table 1.—*Brown and Rhizopus rots in inoculated peaches sprayed or dusted with fungicides and then held 3 and 6 days at 75°F*[a]

Fungus and fungicide treatment	Concentration	Average decay in 3 days			Average decay (cumulative) in 6 days		
		Untreated fruit (check)	Treated fruit	Reduction as percentage of decay in check	Untreated fruit (check)	Treated fruit	Reduction as percentage of decay in check
	%	%	%		%	%	
Monilinia							
Dowicide A spray	0.5	26	2	92	69	27	61
Orthocide 50W spray	1.0	27	6	77	74	21	72
Isothan Q15 spray	0.5	23	7	70	70	27	61
Sulfur dust	94	26	9	65	70	27	61
Rhizopus							
Dowicide A spray	0.5	61	12	80	66	30	55
Orthocide 50W spray	1.0	62	42	32	67	60	11
Isothan Q15 spray	0.5	54	71	−32	60	82	−37
Sulfur dust	94	59	57	3	58	60	−3

[a] Each average based on 16 replicates of 50 peaches. Negative sign before a value indicates increased decay.

decay. Foster (3) reported in 1952 that Orthocide 406 (50 per cent captan) when used as a preharvest spray effectively reduced postharvest brown rot. Results from Isothan Q15 are in agreement with those of Heuberger and others (5), who found that Isothan Q15 used as a postharvest dip effectively reduced brown rot but increased Rhizopus infection. All the materials except sulfur dust caused a diffuse mottle on the surface of some of the fruit in many of the tests. This injury did not extend into the flesh and was considered inconspicuous and of minor importance.

The possibility of reducing the 2 decays by fumigation treatments with 12 different chemicals was investigated, but most of them were unsatisfactory. Among the 12 were 4 chlorinated hydrocarbons reported by Vandemark and Sharvell (9) to give perfect or nearly perfect control of both decays. In a previous study made in this laboratory (7), 2 of the materials, tetrachloroethane and 1,1,2-trichloroethane, caused injury and off-flavor to the fruit and were therefore discarded. Tests of the other 2 for 2 or 3 years are reported in Table 2 in comparison with sulfur dust.

At the 3-day reading, tetrachloroethylene and trichloroethylene had reduced brown rot by a very high percentage, considerably higher than that obtained with sulfur dust (Table 2). Both fumigants had also reduced Rhizopus decay by a high percentage. After 6 days at 75°F, however, brown rot in peaches treated with tetrachloroethylene or trichloroethylene was slightly less or slightly greater than that in untreated fruit. In contrast, both fumigants had reduced Rhizopus rot by more than 50 per cent. Only slight differences in decay reduction or increase were obtained when the materials were volatilized rapidly on a hot plate set at low heat or more slowly at normal air temperature (75°-85°).

Further tests with tetrachloroethylene consisted of fumigation of sulfur-dusted peaches. This combination treatment reduced brown rot after 3 days by a very high percentage, and the reduction was still satisfactory after 6 days (Table 2). At both reading periods, the reduction was greater than that obtained with sulfur dust. The combination treatment also reduced Rhizopus rot by more than 50 per cent at both the 3- and the 6-day reading, but reduction was approximately the same as when tetrachloroethylene alone was used.

Discussion.—To be considered satisfactory, a postharvest fungicide must protect peaches from decay until they are used or are fully ripe. Cravens and Paul (6) studied the time lapse for different phases

Table 2.—*Brown and Rhizopus rots in inoculated peaches fumigated or dusted with fungicides and then held 3 and 6 days at 75°F*

Fungus and fungicide treatment	Concentration	Method of volatilization	Average decay in 3 days			Average decay (cumulative) in 6 days		
			Untreated fruit (check)	Treated fruit	Reduction as percentage of decay in check	Untreated fruit (check)	Treated fruit	Reduction as percentage of decay in check
			%	%		%	%	
Monilinia								
Tetrachloroethylene[a]	1:20,000	Heat	18	3	83	65	63	3
Trichloroethylene[a]	1:20,000	Heat	30	2	93	67	76	—13[c]
Sulfur dust[a]	94%		18	7	61	68	33	52
Tetrachloroethylene[b]	1:20,000	Air	24	0	100	65	55	15
Tetrachloroethylene[b]	1:20,000	Heat	24	4	83	65	64	2
Trichloroethylene[b]	1:20,000	Air	32	1	97	65	68	—5[c]
Trichloroethylene[b]	1:20,000	Heat	32	3	91	65	74	—14[c]
Tetrachloroethylene[b]	1:20,000	Heat	30	1	97	64	63	2
Sulfur dust plus tetrachloroethylene[b]	1:20,000	Heat	30	2	93	64	19	70
Sulfur dust[b]	94%		20	6	70	58	30	48
Rhizopus								
Tetrachloroethylene[a]	1:20,000	Heat	75	16	79	78	35	55
Trichloroethylene[a]	1:20,000	Heat	75	14	81	80	33	59
Sulfur dust[a]	94%		72	71	1	76	77	—1[c]
Tetrachloroethylene[b]	1:20,000	Air	89	23	74	90	40	56
Tetrachloroethylene[b]	1:20,000	Heat	89	22	75	90	38	58
Trichloroethylene[b]	1:20,000	Air	84	15	82	88	36	59
Trichloroethylene[b]	1:20,000	Heat	84	18	79	88	36	59
Tetrachloroethylene[b]	1:20,000	Heat	86	25	71	89	35	61
Sulfur dust plus tetrachloroethylene[b]	1:20,000	Heat	86	22	74	89	44	51
Sulfur dust[b]	94%		85	82	4	91	85	7

[a] Each average based on 12 replicates of 50 peaches.
[b] Each average based on 8 replicates of 50 peaches.
[c] Negative sign before a value indicates increased decay.

of marketing Michigan peaches. They reported that the average time for peaches to reach the consumer was 4½ days and that the consumer kept the peaches 3½ days before use. From their report it would appear that a satisfactory postharvest fungicide should reduce decay for approximately 8 days. In the present report, both 3- and 6-day readings are presented to show the difference in the rapidity of development of symptoms caused by the 2 decay-producing organisms, as well as the duration of decay control by the respective fungicides. Final evaluations of the chemicals tested were based on their performance after 6 days, when most of the peaches were considered fully ripe.

Data presented in a previous report (4) showed that sometimes Rhizopus may cause more postharvest decay than Monilinia on commercial lots of peaches that were not artificially inoculated. To be satisfactory, a fungicide treatment should be effective against both organisms. Of the materials for which data are presented, sulfur dust and sprays of Orthocide 50 Wettable and Isothan Q15 were effective against brown rot only, whereas the fumigants trichloroethylene and tetrachloroethylene were effective against Rhizopus rot only. The spray treatment with Dowicide A and the combination treatment of sulfur dust followed by tetrachloroethylene fumigation reduced both decays by at least 50 per cent.

The materials reported in this paper were tested for fungicidal properties only. It is not known whether they would leave residues toxic to man when used as indicated.

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A NEW NEMATODE GENUS, MELOIDODERA, LINKING THE GENERA HETERODERA AND MELOIDOGYNE [1]

B. G. Chitwood, C. I. Hannon, and R. P. Esser [2]

SUMMARY

A new nematode genus, *Meloidodera*, and the type species, *Meloidodera floridensis*, parasitic on roots of the southern slash pine, *Pinus elliottii*, are described. The nematode is considered to be a link between *Heterodera* and *Meloidogyne*.

Certain collections of pine seedlings and soil sent to this laboratory by R. L. King, Jr., October 12, 1955, from the Florida State Forest Service, Olustee, Florida, were found to contain larval nematodes that were originally identified as "*Heterodera trifolii*?". Since incubation[3] of the roots provided additional larvae in larger numbers, the roots themselves were examined. These were found to contain white female nematodes resembling *Heterodera rostochiensis* Wollenweber 1923 in general body form. Microscopic study disclosed that they were neither *Heterodera* nor *Meloidogyne* but a new genus intermediate between these 2 genera. Emerging females break through the root surface, forming pits in the roots; the neck is internally positioned. No root galls are formed. The new genus apparently belongs in the subfamily *Heteroderinae*, Family *Heteroderidae*.

Meloidodera new genus.—Diagnosis *Heteroderinae:*

[1] Accepted for publication January 9, 1956.
[2] Chief Nematologist, Nematologist, and Laboratory Technician, respectively.
[3] Young, T. W. 1954. An incubation method for collecting migratory endo-parasitic nematodes. U. S. Dept. Agr. Pl. Dis. Rptr. 38: 794-795.

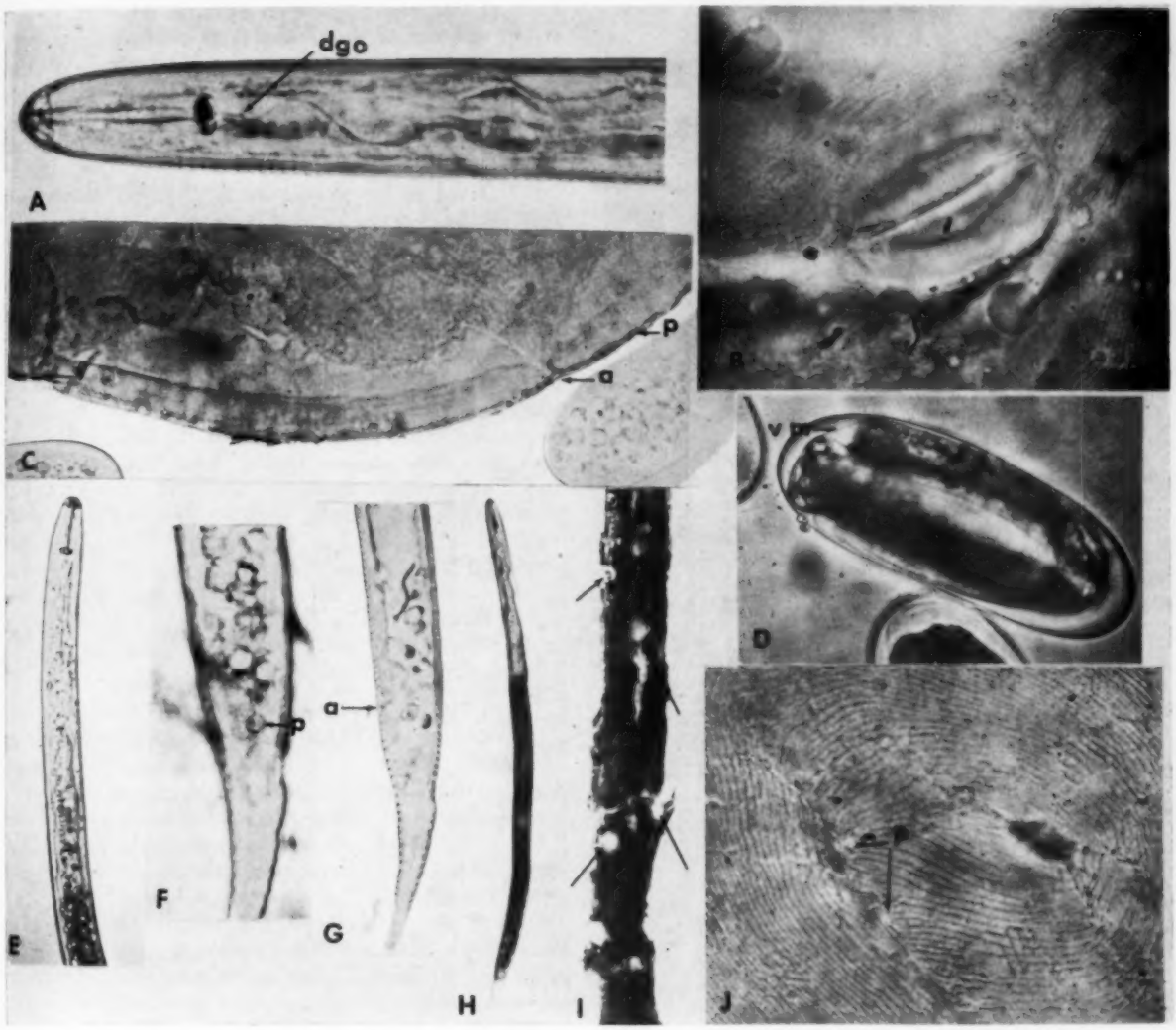

FIG. 1. *Meloidodera floridensis*. A) Anterior end of larva, showing dorsal gland orifice (dgo). B) Surface pattern, vulvar region. C) Lateral view of cyst wall, showing position of vulva (v), anus (a), and phasmid (p). D) Egg containing a larva, showing vitelline membrane (vm). E) Anterior end of larva, showing position of esophageal glands. F) Anal region of larva, showing phasmid (p). G) Caudal region of larva, showing anus (a). H) Larva, full length. I) Root of *Pinus elliottii*, showing emerging females. J) Surface pattern in anal region, showing phasmids (p).

Females resembling *Heterodera rostochiensis*[4] in general body form, skin tough but not colored or with yellowish cast, with internal rows of punctations parallel to annules. Surface markings in form of plain striae about 2 μ wide, resembling *Meloidogyne*. Anus moderately removed from vulva (Fig. 1, C). Pattern in vulvar area somewhat resembling *Meloidogyne hapla* Chitwood 1949[5] (Fig. 1, B; 2, F); also with markings at anus (Fig. 1, J; 2, C); faint indications of partial longitudinal grooves posterior to anus and in cervical region, striae interrupted laterally and sometimes fused laterally. Stylet of female quite long with large knobs similar to *Heterodera* or *Criconemoides* (Fig. 2, E). Eggs retained in female.

Larva (Fig. 1, H) resembling *H. trifolii* but with fewer head annules and larger phasmids (Fig. 1, F).

Type species—*M. floridensis* n. sp.

The genus *Meloidodera* may be distinguished from *Meloidogyne* by the larger stylet in the females, by the hardening and toughening of the body wall, by the separation of vulva and anus, and by the internal "punctation" of the annules. The infective larvae may

[4] Franklin, Mary T. 1951. The cyst-forming species of Heterodera. Commonwealth Agr. Bur., Farnham Royal, England. 147 p.

[5] Chitwood, B. G. 1949. "Root-knot nematodes"—Part I. A revision of the genus Meloidogyne Goeldi, 1887. Helm. Soc. Wash. Proc. 16: 90-104.

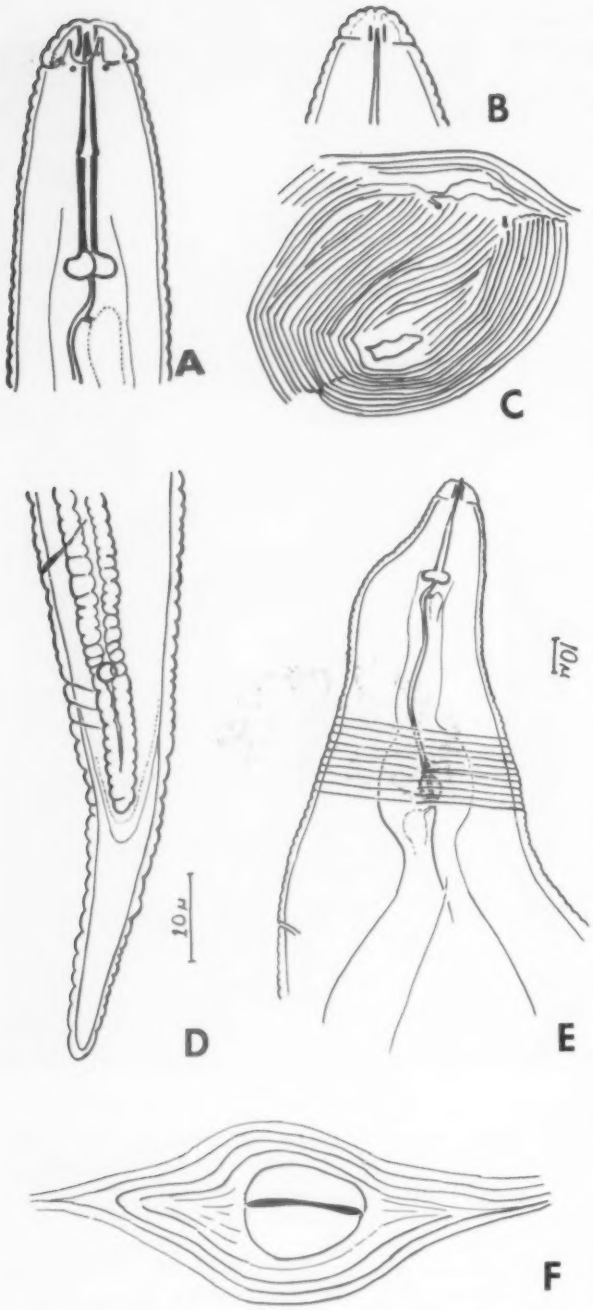

FIG. 2. *Meloidodera floridensis*. A) Head of larva, lateral view. B) Head, female. C) Anal pattern, female. D) Tail of larva, lateral view. E) Esophageal region, female. F) Vulvar pattern, female.

be distinguished from larvae of *Meloidogyne* on the basis of the head annulation (Fig. 2, A), size of the stylet, and size of the phasmids as well as disappearance of the median longitudinal ridge posterior to the anus (Fig. 2, D). *Meloidodera* differs from *Heterodera* in the size of the female stylet, presence of distinct pattern form annules, distance of anus from vulva, and lack of a distinct brown cyst stage. Eggs are retained in the mother as in *Heterodera*, and no root swellings occur as in that genus, but slight swellings do occur with appearance of females (Fig. 1, I).

Meloidodera floridensis n. sp.—*Female*.—Female 500–800 μ long by 220–400 μ wide; head with 3 clear, probably 4 annules (Fig. 2, B); neck region 120–180 μ long; annules about 1 μ wide in anterior region to about 2 μ wide in body region; stylet 35–37 μ long with knobs 2.5 × 8 μ; dorsal gland orifice about 5 μ posterior to base of stylet; bulb 35 × 25 μ; excretory pore near base of neck region, about 130 μ from anterior extremity in specimen with 160 μ neck; vulva on ventral side of body, slightly posterior to middle (450 μ back in specimen 650 μ long); vulva-anus distance, 90–300 μ; anus subterminal, ventral; phasmids 30–40 μ posterior to anus. Eggs varying in size with age of larva up to 115–180 μ long by 55 μ wide. Larva molts once in egg (490 μ long); vitelline membrane present at least in some eggs (Fig. 1, D).

Male.—Not observed from this host.

Larva.—Larva 500–559 μ long by 19–21 μ wide; a, 26–29; b, 6.4–7.1; c, 8.4–10.0; stylet 27–29 μ long; knobs 2–2.5 μ long by 6–7 μ wide, strongly set off; dorsal gland orifice 4–6 μ posterior to base of stylet (Fig. 1, A); head with 4 annules, 5 μ long by 10–11 μ wide (Fig. 2, A); esophageal glands in ventral column extending 90–95 μ posterior to bulb (Fig. 1, E); excretory pore 108–110 μ posterior to head; lateral fields 5–6 μ wide, sublateral ridges striated at least in anal region; lateral areas a declivity in this region; phasmids large, circular, 9–12 μ postanal, sometimes appearing as a pore, sometimes as a disc (Fig. 2, D); anal body diameter 15–16 μ; tail elongate, conoid with blunt tip (Fig. 1, G); postprotoplasmic tip 23–35 μ; striae about 2 μ apart.

Type host.—*Pinus elliottii* Englm., root.

Type locality.—Florida Forest Service, Olustee, Florida, and U. S. Forest Service Nursery, Olustee, Florida.

Cotype specimens.—State Plant Board of Florida, Nematology Collection F-303, F-342.

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MINERAL OILS AS CARRIERS OF UREDIOSPORES OF THE STEM RUST FUNGUS FOR INOCULATING FIELD-GROWN WHEAT[1]

J. B. Rowell and E. B. Hayden[2]

SUMMARY

Two mineral oils (light petrolatum U.S.P. and a 1:1 mixture of light petrolatum plus S/V Sovaspray 100) were effective carriers of urediospores of *Puccinia graminis* var. *tritici* (Eriks. & E. Henn.) Guyot for the inoculation of wheat in the field. Heavy infections were obtained where inoculations with urediospores carried in mineral oil, in water, in 1 per cent aqueous Tween 20, or in talc were followed by incubation under polyethylene tents. With natural conditions of incubation, however, much heavier infections resulted from urediospores applied with mineral oil carriers than with water and talc. Presumably, urediospores applied to plants in mineral oil were more resistant to washing and weathering.

Nonphytotoxic oil fractions of petroleum may be used as carriers in the application of chemotherapeutants for cereal rust control. Recent studies by the senior author of the behavior of urediospores and sporelings of *Puccinia graminis* var. *tritici* (Eriks. & E. Henn.) Guyot in various oils[3], indicated that mineral oils might be effective carriers of spores for inoculating wheat with the stem rust fungus. Further tests were made during the growing season of 1955 on wheat grown in field plots at St. Paul, Minnesota, to compare the effectiveness of inoculum carried in oil, water, and talc. Mineral oils as carriers of urediospores were superior in certain respects to water and to talc and may be useful in making field inoculations of various cereals with rust fungi.

MATERIALS AND METHODS.—Four separate tests were made comparing the effectiveness of 5 treatments for inoculating wheat with urediospores of *Puccinia graminis* var. *tritici*. One-tenth g of freshly collected urediospores of Race 15B (approximately 95 per cent germination on 1.5 per cent water agar) was mixed respectively with 1) 5 ml light petrolatum U.S.P. (i.e. pharmaceutical mineral oil), 2) 5 ml of a 1:1 mixture of light petrolatum plus S/V Sovaspray 100 (an isoparaffinic, low viscosity, spray oil product of Socony-Vacuum Oil Co., Inc.), 3) 5 ml of distilled water, 4) 5 ml of 0.1 per cent Tween 20 (polyoxyethylene sorbitan monolaurate) in distilled water, and 5) 3 g of talc. A noninoculated control was included to measure inoculum drift and to determine the quantity of effective inoculum present in nature. Urediospores in liquid carriers were applied by a glass atomizer using air at 15 lb./sq. in. pressure from a portable gasoline-powered air-compressor. A cyclone-type hand duster[4] was used to apply the uniform mixture of urediospores and talc. The quantity of urediospores and talc used was based on the approximate rate currently used in inoculating plants in field plots at St. Paul, Minnesota.

Each treatment was applied to a 3-ft. section of the row and replicated 3 times on spreader rows of rust-susceptible wheat varieties in the wheat rust nurseries. All treatments were applied in late afternoon. Conditions favorable for infection by the stem rust fungus were provided artificially in 2 tests by covering water-moistened test plots with polyethylene tents. Infection in the 2 other tests was dependent on natural conditions. The severity of infection was estimated in percentages of the surfaces of leaf blades and stems covered by rust according to the modified Cobb scale used by the U. S. Department of Agriculture[5]. According to this scale, the severity of infection is recorded as 100 per cent when 37 per cent of the plant surface is covered by sori.

RESULTS.—Relatively severe infection developed on wheat leaves exposed to inoculum applied with all carriers when the inoculated plants were incubated under polyethylene tents (Table 1). Heaviest infections of leaves and stems resulted from inoculum carried in water. Under these conditions, urediospores were suspended in the water by violent agitation and rapidly atomized onto the plants. Since the water carriers atomized rapidly as coarse droplets, probably less loss from drift resulted than with the talc dust or the fine spray droplets produced by the slower atomization of mineral oil. The severity of infection on stems was noticeably less than leaf infection from inoculum carried in oil. The waxy bloom normally present on wheat stems was completely obscured by the uniform film of oil covering the stems. Possibly the oil

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[2] Plant Physiologist and Plant Pathologist, respectively, Field Crops Research Branch, Agricultural Research Service, U. S. Department of Agriculture, St. Paul 1, Minnesota.

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[5] Melchers, L. E., and J. H. Parker. 1922. Rust resistance in winter-wheat varieties. U. S. Dept. Agr. Bul. 1046.

TABLE 1. *Relative amounts of infection of field-grown wheat inoculated with urediospores of Puccinia graminis var. tritici (Race 15B) suspended in various carriers*

	Percentage infection (modified Cobb scale) of plants inoculated on indicated dates[a]							
	Artificial incubation[b]				Natural incubation[c]			
	Leaves		Stems		Leaves		Stems	
Carrier	May 18	June 3	May 18	June 3	June 3	June 14	June 3	June 14
Noninoculated control	Tr	6	Tr	1	Tr	3	Tr	2.4
Mineral oil	40	33	15	15	38	15	Tr-	0.7
Mineral oil & Sovaspray 100 (1:1)	48	30	8	18	33	24	Tr-	1.7
Tween (1%)	45	42	20	22	15	5	Tr	3.0
Water	50	58	55	42	2	3	Tr	4.0
Talc	28	43	53	32	2	6	Tr	Tr.

[a] In the modified Cobb scale the severity of infection is recorded as 100 per cent when 37 per cent of the plant surface is covered with sori. Tr indicates trace.

[b] Treatments applied to Minhardi wheat (C.I. 5149).

[c] Treatments applied to rows consisting of a mixture of 3 hard red spring wheat varieties: Marquis (C.I. 3641), Ceres (C.I. 6900), and Federation (C.I. 4734).

coating reduced retention of water in adequate amounts on these surfaces to permit most rapid spore germination. When these culms were observed subsequently, it was evident that secondary infections from natural inoculum had occurred freely on these plants. Thus, the oil treatment only temporarily reduced the number of infections that occurred and had not altered permanently the infectability of the variety.

An advantage of oil-carried inoculum of urediospores was particularly evident when infection was dependent on natural conditions. In a preliminary test, a series of the treatments applied to winter wheat on May 18 was not followed by artificial incubation. The weather was extremely hot, dry, and unfavorable for infection until a series of light rains fell during the period from May 26-28. As a result of infection that occurred at that time, a moderate amount of stem rust (10 per cent) developed on the leaves treated with the mineral oil and Sovaspray 100 mixture, and a slight amount (1 per cent) developed on those treated with mineral oil, whereas only traces of rust were present in the remaining treatments. Two additional tests (Table 1) were made comparing the effectiveness of inoculum in the different carriers when inoculated plants were exposed only to natural conditions. In these tests, a 4-in. pot of seedlings of Little Club wheat (C.I. 4066) was placed in each row during treatment and then incubated the same day in greenhouse incubation chambers. Relatively heavy infection resulted from all treatments, indicating the effectiveness of the original inoculum in all carriers. Conditions favorable for infection occurred immediately following the inoculations made on June 3, when 0.6 in. of rain fell. The heavier infection of leaves exposed to inoculum carried in oils indicates that such inoculum withstands washing more readily than spores carried in water or talc. When inoculations were made on June 14, the first favorable incubation period occurred during the period of June 18-19, when 0.16 in. of rain fell and dew was present on plants for 15 hours[6]. Again heavier infection developed on foliage to which oil-carried urediospores were applied than on foliage given any of the other treatments. The tendency for oils to reduce the number of lesions that developed on stems was again evident.

Early rust symptoms were slightly retarded when oil carriers were used, but the effect disappeared during the maturation of pustules. Similar results have been observed with seedlings in the greenhouse as well as with older plants growing in the field. Although the rate of application in the field (5 ml/3-ft. row section) was relatively heavy for oil, there was no apparent injury in any of the oil-treated plots throughout the tests.

DISCUSSION.—Petroleum oils as carriers for urediospores of *P. graminis* var. *tritici* in the field inoculation of wheat have specific advantages. Spores are dispersed readily in oils, and the resulting suspension is more easily maintained than spores mixed with aqueous carriers. Inoculum applied to wheat in oil is more resistant to washing and possibly to weathering than water- or talc-carried spores. Laboratory tests have shown that spores in oil remain viable longer than dry spores and do not germinate until the oil comes in contact with free water[3]. Thus, spores would be unaffected by suspension in oil for the several hours required to inoculate all plants in relatively large field plots. The limitation in numbers of lesions that developed on stems exposed to oil-carried spores might be overcome by reducing the amounts of oils so that only partial wetting of the stems occurs.

The results of these field tests indicate that urediospores carried in oil can be used to inoculate plants in large test plots more uniformly than is presently feasible. Stem rust epidemics in rust nurseries at the Minnesota Institute of Agriculture are induced by inoculating susceptible plants in spreader rows, interplanted among and surrounding test varieties, early in the season and relying on natural conditions for dissemination to, and infection of, the test varieties. Initial inoculum carried in oil might be sprayed over all the test varieties, and conditions favoring infection might be provided by overhead irrigation or natural rains. Uniform and consistent infection might result throughout the nurseries.

[6] Records of the occurrence and duration of dew obtained by a Taylor Dew Meter furnished by Dr. E. B. Lambert.

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HOST RANGE, TEMPERATURE RELATIONSHIPS, AND PATHOGENICITY OF CERCOSPORA LOTI [1]

K. W. Kreitlow and Helen Sherwin Yu [2]

SUMMARY

Cercospora loti, a fungus causing a leafspot of *Lotus uliginosus*, was found in Florida, Georgia, and Maryland. In controlled temperature trials, most cultures of the fungus grew best at 25°C. All varieties and selections of *L. uliginosus* tested were highly susceptible to infection. *L. corniculatus* was intermediate, whereas *L. tenuis* was highly resistant. Of the species of *Lotus* studied, only *L. peregrinus* was immune. Among other legumes investigated, alfalfa and Dixie Wonder pea became infected following artificial inoculation. There was some difference in virulence among single-spore isolates.

In an earlier report[3], a leafspot caused by the fungus *Cercospora loti* Hollós was described on the forage legume *Lotus uliginosus* Schk. Since then, additional information has been obtained concerning the occurrence, host range, temperature relationships, and pathogenicity of the fungus.

The leafspot was first observed in June, 1954, on plants of *L. uliginosus* in a clonal nursery at Beltsville, Maryland. The same leafspot was seen several days later on plants of *L. uliginosus* in a pasture near Alapaha, Georgia. During February, 1955, the leafspot was collected on *L. uliginosus* in forage plots near Gainesville, Florida. Since then diseased leaves on which the fungus was fruiting have been observed at Beltsville, Maryland, from April to November.

Microscopic examination of spores from lesions on diseased leaves obtained at each location revealed the presence of a species of *Cercospora* that most closely resembled *C. loti*, which was described by Hollós on leaves of *Tetragonolobus siliquosus* (L.) Roth (*L. siliquosus* L.) in Hungary[4,5]. To date, the leafspot has not been observed in the field on birdsfoot trefoil, *L. corniculatus* L., a closely related forage species.

SYMPTOMS.—Under both natural and artificial conditions usually 1 to several lesions developed on a leaflet of *L. uliginosus*. Depending upon age and size of the leaflet, each lesion when fully developed was characteristically circular in shape, avellaneous to dark olive[6] in color, 0.5–10 mm in diameter (average 3–5 mm). Earliest symptoms of infection following artificial inoculation occurred within 6 days at 28°C. Initially, the lesions were frequently marginal and ranged in size from pin-point to 1 mm in diameter. At first the lesions were tannish-gray with a slight purple tinge. As they enlarged, they became olive-gray to reddish brown. Maximum development was reached in 12 days. When the fungus was sporulating, the lesions usually developed a grayish tinge. Conidia of Cercospora developed profusely in the lesions. Diseased leaflets shriveled and dropped at the slightest touch, leaving infected plants badly defoliated.

Lesions on petioles and stems were usually reddish brown, and they often extended several centimeters. Sometimes stems were girdled and the leaves withered and fell off. Frequently, diseased shoots died.

MATERIALS AND METHODS.—Radial growth of cultures of *C. loti* was determined at controlled temperatures of 20°, 25°, and 30°C. Five single-spore cultures isolated from *L. uliginosus* growing near Alapaha, Georgia, and 5 single-spore cultures from Beltsville, Maryland, were compared. In addition, a multispore culture isolated from diseased plants at Beltsville and a multispore culture reisolated from a plant of *L. corniculatus* inoculated artificially in a greenhouse were included. The cultures were transferred from agar slants to Petri dishes of Difco potato-dextrose agar. After 12 days, 9-mm disks were cut aseptically with a cork borer from each Petri dish culture. Each disk was placed in the center of a Petri dish containing 20 ml of potato-dextrose agar. Three Petri dishes of each culture were then incubated at each temperature. Rate of growth and other characteristics were observed every 2 or 3 days during the 13-day incubation period.

Inoculum for testing host range and pathogenicity of isolates was prepared from cultures grown in 250-ml Erlenmeyer flasks containing 35 ml of carrot decoction. The decoction was prepared by autoclaving 250 g of chopped carrots in 1000 ml of distilled water for 15 minutes at 15 lb. pressure. The liquid portion was decanted, and 10 g of dextrose added before the medium was dispensed into flasks and sterilized.

After 14 days' growth in the carrot-decoction medium, the cultures were shredded in a Waring Blendor and then sprayed on test plants that were grown from seed in a greenhouse. Noninoculated check plants were sprayed with sterile water. The inoculated plants and

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The authors are indebted to Mr. Paul Henson for seed of the various *Lotus* species tested and to Mr. Albert DePriest for valuable assistance in the greenhouse inoculation tests.
[2] Principal Pathologist and formerly Assistant Pathologist, respectively, Field Crops Research Branch, Agricultural Research Service, U. S. Department of Agriculture.
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[6] Ridgway, R. 1912. Color standards and color nomenclature. Published by the author. Washington. 43 p. [53 pl.].

controls were then incubated in a compartmented moist chamber at 25°C for 72 hours. Following incubation in the moist chamber, the plants were kept in a greenhouse at 28°C where they were subjected to simulated heavy dew produced by a fog-producing humidifier[7].

In the host-range studies, different varieties of *L. uliginosus* and of *L. corniculatus* and other species of *Lotus* were tested for susceptibility to the fungus. In addition, some related hosts and species of other legumes were inoculated. Most species were tested as seedlings (4–6 weeks old) and as older plants (8–12 weeks old). From 4 to 6 plants of each species were inoculated. For inoculum, 4 single-spore isolates from Beltsville collections and 3 single-spore isolates from a Georgia collection were grown separately in the carrot-decoction medium. The isolates from each collection were combined before they were sprayed on duplicate sets of test plants.

To determine whether differences in pathogenicity occurred among single-spore isolates or among isolates from different locations, 7 single-spore isolates were compared on species of *Lotus*, on strains and selections of *L. uliginosus*, and on related species. Three isolates from Georgia, 2 from Maryland, and 2 from Florida were grown separately in the carrot- decoction medium, and each was used to inoculate 2 or more plants of a species or selection. The plants were 8–12 weeks old when inoculated and were examined each day thereafter for symptoms of infection. Although it would have been desirable to test more plants of each species, a more extensive test could not be made because seed of some introduced species was scarce and germinated poorly.

RESULTS.—*Rate of growth of C. loti at different temperatures.*—Most of the cultures grew best at 25°C (Table 1); however, several grew just as well at 30°. At 25°C, most of the cultures were light to dark gray. Light gray to whitish sectors developed in several cultures regardless of temperature. Isolates at 30°C were usually lighter colored than those at lower temperatures. The optimum temperature for growth of most isolates was between 25° and 30°C.

Susceptibility of Lotus and related species.—Among the *Lotus* species tested, *L. uliginosus* and *L. wrightii* (Gray) Greene were most susceptible (rating of 5). All varieties and selections of *L. uliginosus* were equally susceptible. *Lotus scoparius* (Nutt.) Ott. and *Dorycnium rectum* (L.) Ser. (*L. rectus* L.) were less susceptible (rating of 4), whereas *L. hispidus* Desf. (*L. divaricatus* Soland.) and *Dorycnium herbaceum* Vill. (*L. dorycnium* Crantz) were given a rating of 3. The varieties of *L. corniculatus* studied were comparatively resistant to infection (rating of 2). *L. filicaulis* Dur. was rated similarly. The highly resistant species (rating of 1) included *L. ornithopodioides* L., *L. tenuis* Waldst. & Kit., *L. tetragonolobus* L., and *Tetragonolobus siliquosus* (*L. siliquosus*). Of the species examined, only *L. peregrinus* L. was immune to infection.

TABLE 1. *Radial growth of 12 isolates of C. loti incubated 13 days at 3 temperatures*

Isolate	Source	Radial growth in mm[a] 20°C	25°C	30°C
4601	Single spore—Alapaha, Ga.	26.0	34.3	31.3
4602		31.0	41.8	30.3
4603		26.3	37.0	37.3
4604		33.0	42.7	35.0
4605		25.2	40.5	31.7
4689	Single spore—Beltsville, Md.	15.5	20.8	22.1
4588		28.1	33.6	21.0
4589		17.6	24.0	27.0
4590		22.3	30.3	23.0
4591		20.3	26.8	24.3
4671	Multispore reisolate from *L. corniculatus*	18.3	24.5	17.6
4583	Multispore isolate—Beltsville, Maryland	16.5	20.2	20.0

[a] Each figure represents the average of 3 cultures.

Symptoms (rating of 3 to 5) on the susceptible species, including the related species, resembled those on *L. uliginosus*. The lesions were first pin-point in size, becoming noticeable 6–7 days following inoculation. The lesions enlarged rapidly, turning first reddish brown and then grayish when the fungus fruited. In most cases, the diseased leaves wilted and fell off. Reddish brown to ash-gray stem discolorations developed on *L. hispidus* Desf. (*L. divaricatus* Soland.), *L. scoparius* (Nutt.) Ott., and *L. uliginosus*. On *L. scoparius*, the stem lesions elongated up to 20 mm, and some older stems were killed. On the other species, the lesions frequently girdled younger petioles, causing death of the leaflets.

Lesions on *L. corniculatus* and *L. filicaulis* developed slowly; lesions were not distinct until approximately 15 days after inoculation. The lesions were usually small and developed mainly on lower leaves. There was little defoliation. Only a few pin-point lesions developed after prolonged incubation on the highly resistant species (1 rating), including *L. tenuis*.

Susceptibility of other legumes.—Lesions developed in approximately 2 weeks on inoculated leaves of Ranger alfalfa (*Medicago sativa* L.) and Dixie Wonder pea (*Pisum sativum* L. var. *arvense* (L.) Poir.). Only a few lesions developed on inoculated leaves of *Anthyllis vulneraria* L. (Table 2). Infection on soybean and vetch was doubtful.

Lesions on leaves of Ranger alfalfa were reddish brown and varied in size from pin-point to about 3 mm in diameter (Fig. 1, A). Some of the larger lesions developed a grayish sheen when the fungus fruited. Sometimes the spots coalesced, forming irregular discolored areas. Infected leaves turned yellow and abscised.

On leaves of Dixie Wonder pea, the lesions were

[7] Kreitlow, K. W. 1949. A greenhouse bench moist chamber for large scale inoculations. Phytopathology 39: 90-91.

TABLE 2.—*Comparative susceptibility of 10 miscellaneous legumes to C. loti*

Host	Disease rating[a]
Anthyllis vulneraria L.	tr
Arachis hypogaea L. var. Spanish	0
Glycine max (L.) Merr. var. Ogden	0 (?)
Lespedeza capitata Michx. var. *calycina* (Schindl.) Fern.	0
Lespedeza stipulacea Maxim. var. Rowan	0
Medicago sativa L. var. Ranger	2
Pisum sativum L. var. *arvense* (L.) Poir. var. Dixie Wonder	2
Vicia sativa L. var. Doark	0 (?)
Vicia villosa Roth var. Hairy	0 (?)
Vigna sinensis (Torner) Savi var. Chinese red	0

[a] 0 = Immune; 5 = very susceptible; tr = trace.

numerous, pin-point, and reddish brown (Fig. 1, B). They sometimes occurred in patches so that the leaf appeared mottled. Parts or all of some leaves became yellow and wilted; later, they shriveled and fell.

A few reddish brown lesions, ranging in size from pin-point to 2 mm in diameter, developed on leaves and petioles of *Anthyllis vulneraria*. The lesions were somewhat mottled and were confined principally to the upper surface of the leaf. When petioles with suspected lesions were incubated in a moist chamber for 7 days, a few conidiophores and spores developed in the lesions. The paucity of lesions and the sparse development of spores suggest that this host is highly resistant to *C. loti*.

A few small reddish brown spots developed on inoculated leaves of soybeans. The lesions failed to develop further, and the fungus did not fruit in them. Inoculated leaves of vetches became yellowed and shriveled somewhat prematurely; however, no definite lesions or spores were found. It was concluded that both soybean and vetches are highly resistant and are not likely to become infected by *C. loti* under natural conditions.

Comparative virulence among single-spore isolates.—The single-spore isolates tested differed in virulence (Table 3). Culture 4690 from Maryland was most pathogenic, whereas culture 4706 from Florida was least virulent. Both Maryland isolates tested were highly pathogenic. No conclusions were drawn concerning races of the fungus among the 7 isolates studied. Variations in susceptibility among the different species of *Lotus* to the 7 isolates probably could be attributed to genetic variability among the limited numbers of plants available. The apparent discrepancy in results with *Dorycnium herbaceum* (*L. dorycnium*) and *D. rectum* (*L. rectus*) in the host range study and in this test might be explained by the 2 kinds of inoculum used. In the host range study, the inoculum consisted of 2 sets of bulked single-spore isolates from Maryland and Georgia, whereas single-spore isolates were used separately in the present test.

DISCUSSION.—The exact relationship of *C. loti* on *L. uliginosus* in the United States to *C. loti* described by Hollós in Hungary nearly 50 years ago is difficult to establish because none of Hollós' type material was available for comparison. Hollós described *C. loti* from leafspots on *Tetragonolobus siliquosus* (L. *siliquosus*). In the present investigations, *T. siliquosus* was classified as highly resistant. There is a possibility, however, that the few plants of this species tested were more resistant than the specimens collected by Hollós. The demonstrated susceptibility of several species of *Lotus* and the spore and conidiophore characteristics reported earlier suggest that this fungus is similar to *C. loti* as described by Hollós. The prevalence of the

TABLE 3.—*Pathogenicity of single-spore isolates of C. loti to species of Lotus and related hosts*

Host	Disease rating[a] with indicated isolates						
	Georgia			Maryland		Florida	
	4601	4602	4603	4689	4690	4701	4706
Lotus uliginosus P.I. 180249	3	tr	3	5	5	5	1
180172	5	4	5	5	5	5	1
F.C. 23740	4	4	2	5	5	5	1
Beltsville 474	4	5	3	5	5	5	3
Vt. 46–16–3	5	5	5	5	5	5	5
49–13	5	5	5	5	5	5	5
49–14	5	4	5	5	5	5	4
49–17	5	5	5	5	5	5	4
49–17–2	4	4	5	5	5	5	5
49–20–4	4	4	5	5	5	5	3
Lotus hispidus	1	0	1	1	3	1	tr
L. filicaulis	0	tr	tr	1	2	0	tr
L. ornithopodioides	0	0	0	0	1	0	0
L. peregrinus		0		0	0	0	
L. scoparius	3	tr	2	5	5	4	3
L. tetragonolobus	tr	2	tr	0	0	0	tr
L. wrightii	4	4	4	5	5	5	tr
Dorycnium herbaceum	0	0	0	1	1	1	0
D. rectum	tr	tr	tr	0	0	0	tr
Tetragonolobus siliquosus	0	0	0	0	1	0	tr
Anthyllis vulneraria	0	0	0	0	0	0	0

[a] 0 = Immune; 5 = very susceptible; tr = trace.

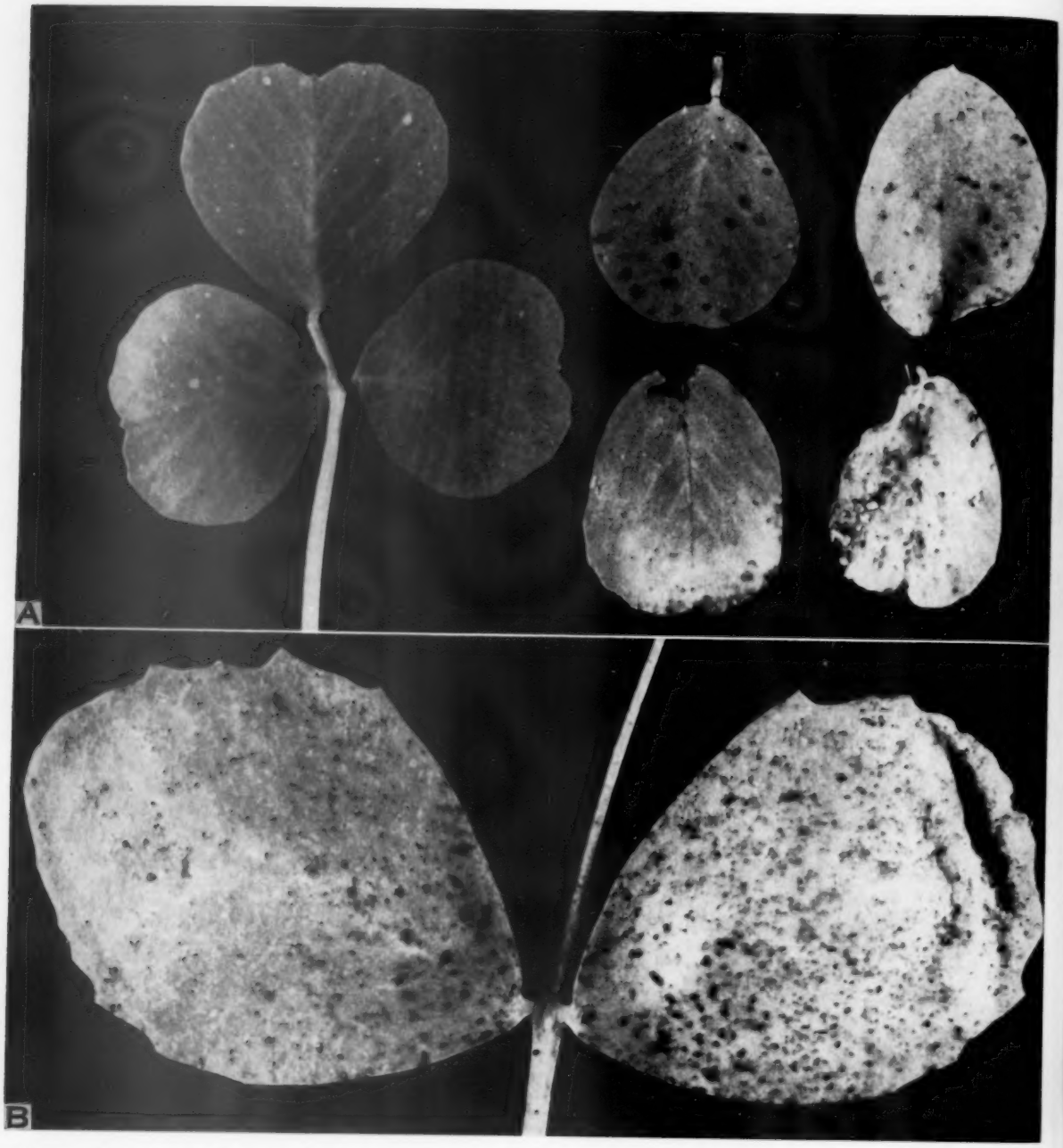

FIG. 1. A) Lesions of *C. loti* on leaflets of alfalfa (right). Left, a healthy trifoliate leaf for comparison. B) Infection on leaflets of Dixie Wonder pea.

disease on *L. uliginosus* along the Atlantic seaboard suggests that *C. loti* should perhaps be considered in the breeding and selection of improved varieties for that region. The complete susceptibility of the varieties and strains of *L. uliginosus* tested does not provide much hope for finding a high degree of resistance within the species. It remains to be seen whether the high resistance among some of the other species of *Lotus* can be utilized through interspecific hybridization with *L. uliginosus*.

Although alfalfa and Dixie Wonder pea were slightly susceptible to infection, there is at present no reason to expect that *C. loti* attacks these hosts in nature.

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ON THE NATURE OF THE MECHANISM OF THE WATER-SOAK METHOD FOR CONTROLLING DISEASES INCITED BY CERTAIN SEED-BORNE PATHOGENS [1]

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SUMMARY

The filtrates from various species and varieties of seed soaked in water for 56 hours at 26°C were examined for organic acid content. All contained volatile acids. These were identified by paper and column chromatography as formic and/or acetic acids, and butyric acid. The only nonvolatile acid detected was succinic.

The germination of spores of *Helminthosporium victoriae* and *Ustilago nuda* was inhibited under anaerobic conditions. Germination of spores of *U. nuda* was also inhibited by steam distillates of barley and oat filtrates and by a solution of formic, acetic, butyric, and succinic acids at various pH levels.

It is a matter of scientific and perhaps of practical interest to understand the mechanism responsible for the effectiveness of the water-soak method for the control of certain diseases that are incited by seed-borne pathogens. The method, which was discovered by Tyner (20, 21), consists merely of soaking seed for a period of time in water. The water-soak treatment has been reported to reduce the incidence of a number of diseases (1, 2, 22, 24).

It is surprising that hundreds of years have passed before this simple method was discovered. It is suspected that there has been prior discovery, but, for one reason or another, the method was not adopted. An instance of accidental discovery (19) may have occurred in 1660, when a ship loaded with wheat was beached near Bristol, England. Plants produced by this seed were noted to be free from bunt, a fact attributed to the NaCl in the sea water. The observation apparently formed the basis for the practice of brining which was used for many years. We can see from the present vantage point that it was probably not the NaCl that was responsible for disease control, but that it is more likely that control was due to the soaking treatment the seed received.

The purpose of this paper is to present a record of experiments aimed at obtaining information on the reason for the effectiveness of the water-soak method. Some constituents of the filtrate from soaked seeds were determined, and spore germination tests with 2 fungi that incite diseases controlled by the treatment are reported. An abstract covering part of this work has appeared (11).

MATERIALS AND METHODS.—*Seed-soaking method.*—In all studies, a ratio (by weight) of 1 part of seed to 5 parts of distilled water was used for the water-soak treatment. Usually 40 g of seed plus 200 ml of water were placed in an open 1-liter Erlenmyer flask and incubated for 56 hours at 26°C. Seeds were removed with a coarse screen and the filtrate (usually pH 5–6.5) used immediately or adjusted to pH 1 with H_2SO_4 and stored at 2°.

Extraction of organic acids.—Fifteen-ml aliquots of the seed filtrates were steam distilled by the method of Friedemann (6). For use in chromatographic or spore germination studies, the distillate was adjusted to pH 7 with NaOH before concentration *in vacuo* to the desired volume.

Thirty-ml amounts of filtrates at pH 1 were extracted continuously with peroxide-free diethyl ether by means of Kutscher-Steudel type extractors. The ether was evaporated from the extract *in vacuo*, leaving an aqueous residue, which was used for chromatographic studies.

Volatile acid determination.—Volatile acids were noted qualitatively by means of paper chromatography, using solvent A of Jones et al (9). A circular paper technique (cf. 16) was used; papers were 15 cm in diameter and equipped with central cotton thread wicks. Samples were spotted in a circle 15 mm from the wick. Although a number of salts of formic and acetic acids were tried, and the system changed in several ways, these 2 acids were not separated on paper. The R_f values for the various acids approximated those reported by Jones et al.

Quantitative determinations of volatile acids were made by column chromatography using a 4-g column of 100-mesh Mallinckrodt silicic acid (AR). The silicic acid was ground with 2 ml of H_2O and washed into a glass column tube (1 cm inside diameter) with solvent I (subsequently described). Air, at 3 lb. per sq. in., was used to pack the column, which was about 10 cm high when packed. Dry silicic acid (0.6 g) in dichloromethane was applied to the top of the column. To 0.2 ml of the sample (Na salt), sufficient 10 N H_2SO_4 was added to lower the pH to approximately 1. The adjusted sample was quickly added to the column. Water (about 0.3 ml) and dichloromethane were used as washes for the sample vessel. Three developing solvents were employed successively; from 50 to 60 fractions

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of 1.5 ml each were collected and titrated (16) to the phenol red end-point with 0.01 N NaOH. Solvent I was dichloromethane equilibrated with water. With this solvent, butyric acid was usually collected in fractions 4–19. Solvent II, added when butyric acid had been collected, was 20 per cent (by volume) 4-methyl-2-pentanone in dichloromethane, the mixture being equilibrated with water. This solvent usually removed acetic acid in fractions 24–40. When acetic acid had been collected, solvent III, 4-methyl-2-pentanone equilibrated with water, removed formic acid in the final fractions. Identities of acids were determined by comparing their elution chromatograms with those of reagent volatile acids and by means of paper chromatography.

Nonvolatile acid determination.—These acids were estimated by paper chromatography by the method of Scott (16). When Scott's solvent B was used, the amount of succinic acid in a given ether extract was estimated by visual comparison of the size and density of the extracted succinic acid "spot" with spots produced by graded amounts of chromatographed reagent succinic acid.

Spore germination methods.—In spore germination studies, 3 drops of a spore suspension were placed on a microscope slide. Drops were confined by rings of silicone (DC Antifoam A, Dow Chemical Co., Midland, Michigan), which did not influence germination. For tests in air, slides were placed in Petri dishes containing moist filter paper. Germination in nitrogen was accomplished by placing slides (usually 3–5) on a wire screen rack in a 15-cm (inside diameter) desiccator, the bottom of which contained about 100 ml of 1 N NaOH plus 5–10 g of pyrogallol. The desiccator was provided with a perforated tube that vented water-washed N_2 near the surface of the alkaline pyrogallol and under the rack. An exhaust was provided in the desiccator lid. The assembly was flushed several times with N_2 after the slides were added. The temperature was 24–26°; germination results were recorded after 18–24 hours.

Spores of *Ustilago nuda* (Jens.) Rostr. were collected from the smutted heads of barley plants and stored at 2°; all experiments were made within a 4-month period. Tween 20 (sorbitan monolaurate, polyoxyalkylene derivative; Atlas Powder Company, Wilmington, Delaware), when used at 0.02 per cent (by volume) as a surface active agent, permitted good dispersion of spores and was used in all tests. With this material, germination (usually over 80 per cent) was better than when it was omitted. Final concentration of spores in drops was about 10^6 per ml.

Spores of *Helminthosporium victoriae* Meehan & Murphy were harvested in distilled water 1–2 weeks after seeding tube slants of potato-glucose agar. Incubation temperature was 26°; the spore concentration was about 5×10^4 per ml.

RESULTS.—One of the first hypotheses as to the mechanism of disease control centered about antibiotics. Indeed, Wallen and Skolko (24) had suggested that such substances were responsible for the effectiveness of the seed-soak method for the control of the Ascochyta seedling blight of pea. We were led, therefore, to examine seed filtrates by means of the Glomerella assay (13) for the detection of diffusible toxicants such as antibiotics. Nearly all filtrates produced inhibition zones; however, these were vague and transitory. Extraction of filtrates at pH 1 with ether removed the inhibitory product (extraction at pH 7 or 9.5 did not). When the ether was removed *in vacuo*, there remained an aqueous yellow-brown solution which produced large inhibition zones. Inhibition was complete throughout the zone at first, but in 4–5 days the zone became overgrown with the assay organism, growth progressing inward from the edge. Organic acids were clearly implicated by the extraction technique; the odor indicated that butyric acid was present. Consequently, various concentrations of C_1–C_9 saturated aliphatic acids were assayed. All produced zones, and overgrowth proceeded inward as with the ether extract. These results indicated that further acid analysis was desirable. It was suspected that ethanol and butanol might be produced, but tests (14) with filtrates from 4 seed lots indicated that ethanol was present at less than 0.1 per cent. Butanol was not detected.

Volatile acid production during the soaking treatment.—Six lots of barley seed, 1 of wheat, and 1 of oats were soaked as usual. There were 3 flasks for each lot. A portion of each filtrate was steam distilled. All filtrates contained volatile acids, and there were significant differences in amounts produced among the lots (Table 1).

Filtrates from lots 1, 4, 6, 8, and 9 (Table 1) were extracted with ether for 4 hours. Also, filtrates from lots 2, 3, and 9 were extracted for 3 days. Extracts were tested by paper chromatography, and each sample showed the presence of butyric acid and of formic and/or acetic acids. When the neutralized steam distillate was concentrated to 1/30 of the original volume and then tested by paper chromatography, the

TABLE 1.—*Steam-distillable acids produced during the soaking treatment for the control of various seed-borne pathogens*

Seed lot	Crop and variety[a]	Average milliequivalents of acid in the filtrate from 40 g of seed[b]
1	Barley, Oderbrucker	0.48
2	Barley, Kindred	1.00
3	Barley, Moore	1.00
4	Barley, Kindred	0.46
6	Barley, Kindred	0.79
7	Barley, Moore	0.58
8	Wheat, Henry	0.54
9	Oats, Vicland	1.29

[a] Lots 1 through 4 were grown at 4 different locations, 6 and 7 at a fifth, and 8 and 9 at a sixth location in Wisconsin.

[b] LSD, 0.01, = 0.11 milliequivalents.

presence of these acids was verified in filtrates from all seed lots.

Portions of filtrates from lots 2, 3, and 9 (Table 1) were steam distilled and the concentrated distillates passed through silicic acid columns for quantitative estimation of volatile acids. Formic, acetic, and butyric acids were found in all 3 samples (Table 2).

Nonvolatile acid production.—Filtrates from 2 barley and 1 oat sample (lots 2, 3, and 9) were extracted with ether for 3 days and the ether extracts tested by paper chromatography for the presence of nonvolatile acids. A number of trials using all 3 solvent systems (16) were made. The only acid that could be detected was succinic, and this was found in all samples at a concentration of about 1.5 mg in the filtrate from 40 g of seed. Three additional samples of filtrates from barley lots 4, 5, and 7 were extracted with ether for 4 hours and tested as above. The only acid that could be detected in these samples again was succinic. Previous experience had shown that the long extraction period virtually freed the sample of ether-soluble acids; the shorter period was sufficient to remove the volatile acids and fumaric, glutaric, α-ketoglutaric, lactic, pyruvic, and succinic acids, among others.

Spore germination tests.—Studies were made on the inhibition of germination of spores of 2 fungi that incite diseases that have been reported to be controlled by the water-soak method. *U. nuda* is carried in the embryo deep within the seed, and *H. victoriae* is believed to be confined to the surface and outer layers. Germination tests with spores, rather than with hyphae, were made as a matter of convenience; it is realized that with these 2 fungi the hyphal form is present in or on the seed.

1) *U. nuda.*—The germination of spores of *U. nuda* under aerobic and anaerobic conditions was studied in 12 tests. In air, germination was 75–90 per cent, but in an N_2 atmosphere it was entirely prevented in 7 tests and much reduced in the remainder (< 30 per cent). Furthermore, the germ tubes of spores that did germinate were shorter than those from spores germinated under aerobic conditions. The N_2 treatment influenced later germinability. This was shown in tests in which spores were placed in N_2 for 18–24 hours, after which the circumambient fluid was replaced with fresh water. Germination was less than 50 per cent in each of the 3 tests. In 2 tests, germination after 18–24 hours in N_2 was also reduced when the germination fluid was not replaced.

The steam distillate from an oat filtrate was used for germinating spores. This material was concentrated to the volume of the filtrate from which it was derived. In 5 tests with the distillate at pH 5 or 7 there was no germination in air or in N_2. In air, a distillate (pH 7) from a barley filtrate permitted an average of 23 per cent germination.

A solution of acids was prepared in the approximate proportions noted in 2 barley and 1 oat filtrate. "Synthetic" contained per liter: 98 per cent formic acid, 0.05 ml; glacial acetic acid, 0.12 ml; *n*-butyric acid, 0.18 ml; and succinic acid, 15 mg. When this solution was used as the germination medium, the average percentage germination (3 tests) at various pH levels was as follows: pH 4, < 1; pH 5, 55; pH 6, 85; pH 7, 86; water (control) 92. Four parallel tests using "synthetic" with 30 mg per l of succinic acid instead of 15 mg yielded the following data (percentage germination); pH 4, < 1; pH 5, 16; pH 6, 56; pH 7, 64; water (control), 86. These results indicate that *U. nuda* spores are inhibited by "synthetic" at low pH levels, and that the steam distillate contains inhibitory substances in addition to the ones in "synthetic".

2) *H. victoriae.*—The steam distillate of the oat filtrate evidently supplied nutrients for the aerobic germination of this fungus, since at pH 5 and 7, average germination was 80 and 94 per cent, respectively; in comparison, germination in water (pH 6.2) was 23 per cent (3 tests). In terms of germ tube length and amount of branching, growth was poor in water, better in the distillate at pH 5, and best in the distillate at pH 7. Germination and growth in N_2 in the same media was less than in air. In parallel experiments using "synthetic" at different pH levels, aerobic germination at pH 4, 5, and 6 was below that in water; at pH 7 germination was 38 per cent. While these tests need further elaboration, it is concluded that in contrast to *U. nuda*, *H. victoriae* is strongly stimulated by materials in the distillate. Like *U. nuda*, however, *H. victoriae* appears sensitive to reduced O_2 tension.

TABLE 2.—*Volatile acids produced during the soaking treatment, as determined by column chromatography*

Acid	Milliequivalents of acid in the filtrate from 40 g of seed of the indicated lots[a]		
	2	3	9
Acetic	0.43	0.21	0.53
Butyric	0.34	0.51	0.24
Formic	0.06	0.14	0.27
Propionic	0.02	0.0	0.0

[a] The material chromatographed was from 1 of the 3 replicate steam distillates (Table 1) made for each lot of seed.

DISCUSSION.—In the present work, characteristic fermentation products were produced when seed of various small grains were soaked in water. All samples, whether of oats, barley, or wheat, yielded filtrates containing significant quantities of formic and/or acetic, butyric, and succinic acids. The organic acids are believed to be produced as the result of the intense anaerobic metabolic activity of microorganisms that develop during the soaking process. The characteristic nature of the fermentation, particularly with respect to the production of formic and succinic acids, suggests that whenever small grain is soaked in water, certain organisms, probably anaerobic acid-producing bacteria, eventually predominate. Those organisms naturally present on the seed are conceived to serve as inoculum for the fermentation. Thus, the soaking process seems to be not unlike other plant-material fermentations

generated by epiphytic microorganisms, e.g. in silage or pickle production.

Bacteria have been demonstrated to be present in large numbers on small grain seed (4, 7, 8, 12, 17, 18). The weight of this work points to the existence of a more or less constant bacterial population with respect to species. Our findings add evidence for this view. Actually, of the species present on grain seed, but a fraction may be important in the fermentation that takes place during soaking. The inhibition of bacterial growth provided by certain antibiotics added to the water used for the soaking treatment did not alter the effectiveness of the method (1), presumably because the inhibited species were not important in the fermentation. Saprophytic fungi may be present also (15), but it seems likely that they, like the fungal pathogens, would be inhibited and be of little consequence in the fermentation.

The nature of the control of disease by the soaking treatment was investigated in these studies. It appears to be linked with the fermentation process. Anaerobiosis is concluded to be one of the important factors. The measurement of the oxygen tension in the seed areas likely to harbor pathogens presents technical difficulties; the nature of the fermentation during the soak indicates that it is low. Experiments showed that the absence of oxygen inhibited both *U. nuda* and *H. victoriae;* with *U. nuda*, a period of anaerobiosis affected the subsequent germinability of spores. In view of the general requirement of O_2 by fungi, the anaerobic conditions during the soaking treatment could be expected to inhibit diverse fungi, including pathogens and nonpathogens alike.

In attempting to explain the reason for the effectiveness of the water-soak treatment, the fact that materials inhibitory to the growth of fungi are produced must also be taken into account. The identified volatile acids are known to possess fungicidal activity. Acetic acid has been shown to be effective as a seed soak in the control of the bacterial canker of tomato (3). In the soaking treatment of small grains, the acid concentration may be higher within the seed than in the filtrate because of the proximity of great numbers of bacteria actively decomposing carbohydrates and other materials. The concomitant increase in hydrogen-ion concentration in the seed would be expected to be inhibitory to *U. nuda*, as demonstrated in experiments with "synthetic"; other fungal pathogens are also sensitive to acid conditions (e.g., 10). Inhibition by fermentation products such as organic acids conceivably could be induced or increased by anaerobic conditions, as has been reported for tubercle bacilli (5).

The above considerations have not included the possibilities of disease control being the result of the production of antibiotics (as defined by Waksman (23)) or as a result of the products of the metabolism of the seed itself. We would rule out neither alternative. The unidentified toxic material of the steam distillate may indeed be antibiotic in nature. The evidence here presented, however, points to the importance of anaerobiosis and to the production of organic acids during the soaking process. We view the present study as an attempt to assess some of the factors in what may well be a complex situation. Information may be gained from seed-treatment tests with the ether extract, the distillate, and the organic acids. These tests are at present in progress.

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PATHOGENICITY ON SUGARCANE AND HOST PLANT STUDIES OF A SPECIES OF TYLENCHORHYNCHUS [1]

Wray Birchfield and W. J. Martin [2]

SUMMARY

An ectoparasitic nematode of the genus *Tylenchorhynchus* was found associated with diseased sugarcane roots in Louisiana. In the presence of large populations of the organism, the symptoms of disease were abnormally blunt, irregular, and sparse roots. Although this nematode could not be cultured on organic or synthetic media containing several soil fungi, large populations developed when it was placed in steam-sterilized soil in which sugarcane was grown. Furthermore, symptoms of disease in such plants were similar to those found in the field. Typical symptoms resulted from infestation with nematodes either in large masses as they were obtained from soil screenings or in small numbers relatively free of other organisms. These results are interpreted to mean that the nematode was both parasitic on and pathogenic to sugarcane.

The nematode was found to reproduce on 2 varieties of soybean which are used in the sugarcane crop-rotation program, and on Johnson grass, which is a noxious weed in sugarcane fields of Louisiana. Other hosts included rice (variety Zenith) and sweet potato (variety Porto Rico).

An undescribed nematode, subsequently named *Tylenchorhynchus martini* Fielding, 1956 (6), was first observed to be associated with the roots of sugarcane near Baton Rouge, Louisiana, in 1951 (1, 2, 7). The stubby, sparse, and deteriorated conditions of the root systems of sugarcane growing in its presence indicated the possible role of this nematode in pathogenesis. Further examination showed that it was abundant in the sugarcane area of Louisiana. This organism was also found in soil obtained from the sugarcane fields of Florida.

Since other species of the genus *Tylenchorhynchus* have been reported as plant parasites (8, 9), it was considered important to study the pathogenicity of this new species. The purpose of this paper is to record the results of pathogenicity and host plant studies upon this species of *Tylenchorhynchus*.

PATHOGENICITY STUDIES.—The soil used in pathogenicity tests of this nematode on sugarcane was steam-sterilized for 2 hours at 20 lb. pressure in 12-in. clay pots. These pots were placed on greenhouse benches and spaced to avoid contamination. The nematodes for the experiment were collected from sugarcane soil by a modification of the Baermann funnel technique as described by Christie and Perry (5).

Infestations were made in 2 ways: 1) large numbers of nematodes and 2) small numbers relatively free of other organisms were placed on sugarcane roots propagated from seed pieces. A dental pulp-needle was found to be an effective instrument for selecting individual nematodes.

The sugarcane variety C.P. 29-116 was propagated from seed pieces (portions of a sugarcane stalk, each containing 1 node), from shoots (excised from seed pieces after root formation), and from true seed. The seed pieces were dipped in a 1:500 solution of mercuric chloride for 3 minutes and rinsed well in sterilized water before planting. One plant was grown in each pot, with 6 pots of each treatment. The sugarcane was allowed to grow 2–8 months, after which each plant was removed and the green weights of the tops and roots of each recorded. The nematode population in the entire pot of soil was estimated at the time the plants were removed. Population estimates were based on pint soil samples. These estimates were made with the first 10 ml of water that drained into 50-mm petri dishes from each Baermann funnel. The nematodes on 10 per cent of the surface area of each dish were counted with the aid of the low-power magnification of a compound microscope.

Nematode infestations in mass.—After it was found that this nematode could not feed and reproduce on

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artificial media in which some of the common soil fungi also were grown, an experiment was completed to determine whether it could feed and reproduce on sugarcane growing in steam-sterilized soil.

Approximately 1500 nematodes were added to each of 6 plants of sugarcane sprouting in steam-sterilized soil. Six nematode-free plants grown under the same conditions served as the controls. At the end of 3 months, the average height and weight of the plants growing in the presence of the organism were less than those of the controls, although the differences were not of statistical significance at the 5 per cent level of probability. Fourteen days after removal of the first crop, a second crop was planted in the same soil. In this case, the differences between the green weights of infested and noninfested roots were statistically significant at the 5 per cent level. The roots of plants grown in the presence of the nematodes were more sparse, more stubby, and more deteriorated than roots of the controls (Fig. 1). The former had differentiated a thick cortical tissue common to aged sugarcane roots, causing them to appear darker than the controls. The "set roots" or the primary roots at the nodes had decomposed completely in the presence of the nematodes, whereas those of the controls remained relatively healthy. The population was estimated to range from about 600 to over 6400 nematodes per plant, averaging approximately 2600. When these nematodes were observed, they were active in all stages of development and apparently well adapted to the environment.

The possibility existed that pathogenic fungi might have been introduced with the nematodes. Therefore, over 300 tissue isolations were made from both the nematode-inoculated plants and the controls. Root tips were washed for about 12 hours, dried between pieces of sterile blotting paper, and plated on water agar. Fungi that started growth on the water agar were transferred to potato-dextrose agar and identified. This technique had been used successfully to isolate *Pythium arrhenomanes* Drechs., known to cause a sugarcane root rot (4); however, *P. arrhenomanes* was not isolated in these tests. The fungi that were isolated were common to plants in both the nematode-infested soil and the controls. Further inoculation tests with the nematode and *Pythium arrhenomanes* separately and together indicated that the pathogenic effects that they caused on sugarcane were independent.

Nematode infestations from "hand-picked" specimens.—When the nematodes, in mass as they were obtained by use of the Baermann funnels, were placed on sugarcane, other parasitic-type nematodes as well as fungi were included in the inoculum. In order to reduce contamination to a minimum, "hand-picked"

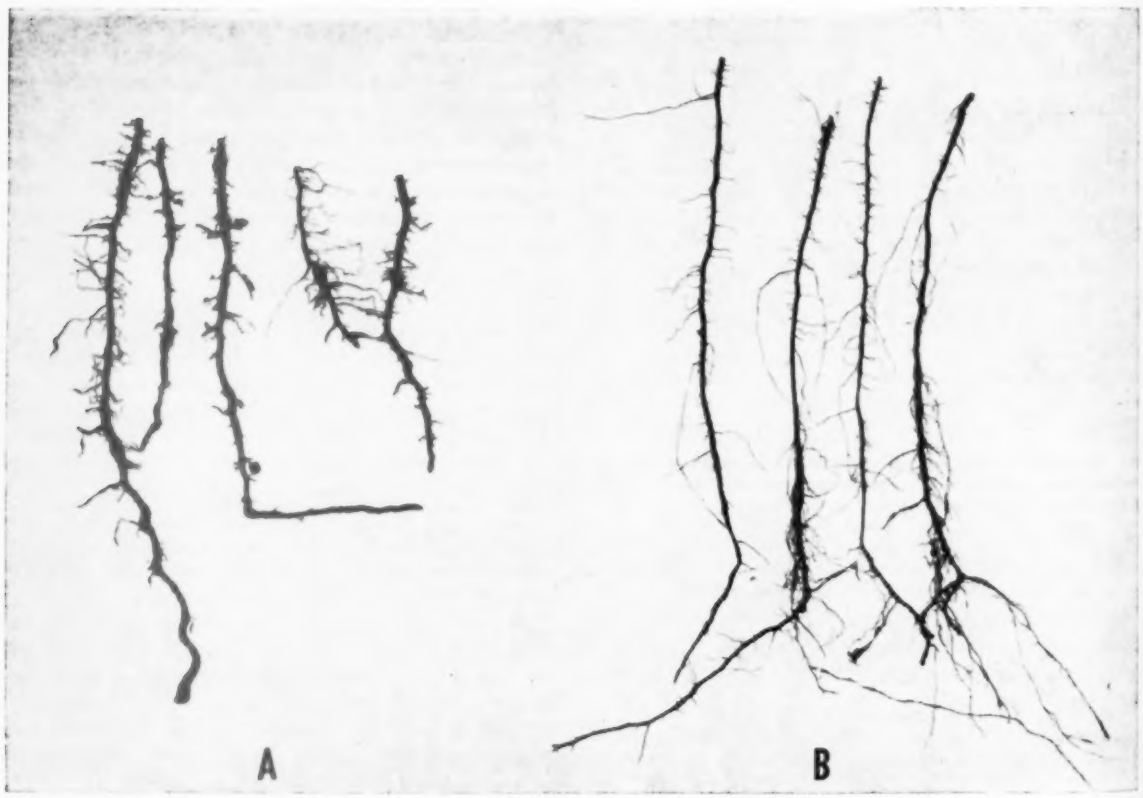

FIG. 1. A) Sugarcane roots grown in steam-sterilized soil artificially infested with *Tylenchorhynchus* sp. B) Sugarcane roots grown in steam-sterilized soil free of nematodes.

specimens were used in 2 experiments. In the first, 50 mature females were added to each of 6 pots of steam-sterilized soil after they had been lifted from the container by means of a splint (with the aid of the stereoscopic microscope) and identity confirmed by means of the compound microscope. The test was started on December 7, 1952, and plants were removed for examination on April 30, 1953.

The root systems of those plants grown in the presence of the nematodes were similar to those in the previous tests. In addition, secondary and tertiary rootlets were absent where the nematodes were present. The remaining primary roots were blunt and malformed, appearing ragged and stubby. The average green weights of plants grown in the presence of the nematodes were less than those of the controls, but the differences were not statistically significant. The nematodes increased to numbers averaging over 11,000 per plant.

A second planting was made in the same soil, which by then contained a large, well-established nematode population. On examination at the time of harvest, symptoms of the disease were as described previously. Differences in green weight between plants in infested and in noninfested soil were not statistically significant (Table 1).

The rapidity of reproduction of this nematode on sugarcane in steam-sterilized soil suggested that small numbers of nematodes were capable of establishing large populations; therefore, in the second experiment 60 mature females were selected and their identity verified, and 10 were added to each of 6 pots of steam-sterilized soil in which 1 plant was growing per pot. During the next 8 months, the nematode population increased from the initial 10 to about 5000 nematodes per plant. Typical symptoms resulted. The differences in green weights between the nematode-infested plants and the controls were statistically significant at the 5 per cent level, indicating that the nematode was not only parasitic upon but pathogenic to sugarcane.

Infestation of excised sugarcane shoots and seedlings.—The possibility existed that the large amount of reserve food in the seed pieces used to propagate sugarcane prevented a full expression of the diseased condition. Therefore, tests were made with the nematodes placed on sugarcane propagated from excised shoots and seedlings.

When young shoots from the old seed piece had formed the first node and consequently the first latent primary roots, the shoots were excised and grown in steam-sterilized soil. Eight plants were established by July 13, 1953, 4 of which were inoculated with 600 nematodes per plant and 4 of which served as controls. These nematodes were the progeny of the "hand-picked" specimens that had been propagated on sugarcane growing in steam-sterilized soil. When the plants were removed after about 6½ months, the symptoms of disease were similar to those in previous experiments, but the differences in green weights between the plants infected with the nematodes and those of the controls still were not outstanding. The nematode population, however, had increased to more than 6000 per plant.

In another attempt to determine pathogenicity of *Tylenchorhynchus martini*, sugarcane propagated from seeds was inoculated when in the seedling stage. When the seeds had germinated in steam-sterilized soil, about 1000 nematode specimens were placed in each of 6 pots containing 1 vigorous seedling. The average green weights of the tops and roots of the seedlings grown in the presence of the nematodes were less than that of seedlings grown without the nematodes, but these differences were not so great as those in previous tests and were not statistically significant. A very large population of nematodes had built up from the original number placed in the pots (Table 1).

Host-plant studies.—In attempts to determine a source of resistance, nematodes were placed on 13 varieties of sugarcane, and records of reproduction and green weights were obtained as before. On the basis of reproduction of the nematode, none of the varieties showed outstanding resistance. The most promising varieties were Co. 281, P.O.J. 213, and *Saccharum spontaneum* L. of India. Typical root symptoms were observed on the majority of the varieties tested; however, in some cases, it was difficult to

TABLE 1.—*Nematode population of soil infested with Tylenchorhynchus by various methods and weight of tops and roots of sugarcane grown in the soil*

Method of propagation	Growth period (days)	Number of nematodes		Green weights (g)[a]			
				Tops		Roots	
		Deposited[b]	Recovered	Control	Infested	Control	Infested
Seed pieces	98	1,500(M)	1,200				
Seed pieces	126	1,200[c]	2,600	41	37	18	14[d]
Seed pieces	112	50(P)	11,000	48	47	12	6
Seed pieces	142	11,000[c]		122	83	71	60
Seed pieces	238	10(P)	5,000	150	71[d]	76	61
Excised shoots	169	600(M)	6,500	211	144	91	89
Seeds	264	1,000(M)	21,000	96	77	67	47

[a] Mean of 6 plants.
[b] M = Nematodes in mass were used in making infestation; P = hand-picked specimens used.
[c] Soil used in test immediately above was replanted for this test.
[d] Differences between infested and control plants were significant at the 5 per cent level.

TABLE 2.—*Number of nematodes recovered from steam-sterilized soil infested with Tylenchorhynchus and planted to various plant species*

Test plants	Growth period in days	No. of nematodes	
		Deposited	Recovered[a]
Johnson grass (*Sorghum halepense* (L.) Pers.)	112	100	2000
Zenith variety rice (*Oryza sativa* L.)	107	125	280
Unit I Porto Rico sweet potatoes (*Ipomoea batatas* Lam.)	107	300	400
Pelican soybeans (*Glycine max* Merr.)	104	300	130
Acadian soybeans (*Glycine max* Merr.)	110	300	250
White Tuxpan field corn (*Zea mays* L.)	106	300	40
Deltapine 15 cotton (*Gossypium hirsutum* L.)	107	125	10
White trumpet Easter lily (*Lillium longiflorum* Thunb.)	120	100	30
Common Ligustrum (*Ligustrum vulgare* L.)	112	300	0
Sweet Clover (*Melilotus indica* All.)	95	300	40

[a] Estimate of the number of nematodes in a pint of soil made up of eight 6-in. cores.

distinguish between the infested plants and the controls. The green weights of the checks generally were greater than those of the infested plants, but the correlation of the magnitude of these differences with the rate of reproduction of the nematode was doubtful.

Soil samples from the rhizosphere of several weeds growing in sugarcane soils contained this nematode in some cases but not in others. In further tests, such plants were grown in steam-sterilized soil and infested with a fixed number of nematodes from sugarcane. The plants were allowed to grow for approximately 110 days, after which estimates of the nematode populations were obtained. The results indicate that Johnson grass and rice (variety Zenith) were very favorable hosts (Table 2). Pelican and Acadian varieties of soybean and Unit I Porto Rico sweet potato were fairly good hosts, whereas Deltapine 15 cotton, white Tuxpan field corn, common Ligustrum, and sweet clover failed to allow substantial reproduction of this nematode. It was doubtful that Easter lily was a host plant.

DISCUSSION.—Although "stylet bearing" nematodes have been suspected of parasitizing sugarcane for a long time, few critical inoculation experiments have been reported in the literature. The pathogenicity of any nematode on sugarcane has never been established. Koch's postulates of proof were applied in a practical sense with a nematode instead of with bacteria as these postulates were originally intended. This organism was also placed on sugarcane with the virulent fungus *Pythium arrhenomanes*. Results of these studies were interpreted to mean that nematodes might cause symptoms of a disease without the presence of fungi or bacteria ordinarily considered as pathogens. However, these tests showed the difficulty involved in measuring the expression of symptoms as determined by green weights where an ectoparasitic nematode was suspected, even though the host plant was propagated by various methods. It seems probable that this organism might lower sugarcane yields under field conditions where vigorous root systems are required for rapid and efficient adjustment to a changing environment.

Host-plant studies indicated that *Tylenchorhynchus martini* feeds on a wide variety of plants. Among these is Johnson grass, a most noxious weed in fields of sugarcane in Louisiana. This weed probably supports a high population on fallow lands. Pelican and Acadian varieties of soybean, commonly used in the rotation program, should be replaced by a resistant or immune variety. The large number of sugarcane varieties used in the breeding program showed very little resistance. This indicated the difficulty of selecting a highly resistant or immune variety that would have the many desirable agronomic characteristics required today. This organism is most probably of economic importance on rice, sweet potatoes, ornamentals, and other plants in Louisiana and Florida and perhaps in other parts of the southern states and Texas.

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FACTORS INFLUENCING FORMATION OF SCLEROTIA BY RHIZOCTONIA SOLANI [1]

G. B. Sanford [2]

SUMMARY

Production of sclerotia of *Rhizoctonia solani* Kühn in steam-sterilized and natural (nonsterilized) soil was compared with that on nutrient agar. A slightly larger number of sclerotia developed in natural soil than in sterilized soil. When a small quantity of natural soil was incorporated in the sterilized soil, however, about 6 times as many sclerotia were produced as were formed either in natural soil or in natural soil amended with sodium nitrate. Sclerotia also developed on such substrates as glass, potato tubers, straw, wood, and stone.

Increased production of sclerotia is related to condensation of the moisture of the air in the soil rather than to total water content of the soil. Approximately 3 times more sclerotia formed in soil at 61° than at 75°F. Isolates of *R. solani* differed in ability to form sclerotia. *Trichoderma lignorum* (Tode) Harz inhibited sclerotia formation in sterilized soil and in natural soil amended with cornmeal but not in natural soil alone. The possibility that certain chemicals produced by associated fungi in soil or by *R. solani* on nutrient agar may initiate the barrel-type cells of sclerotia is discussed.

In a previous publication (8), the possible importance of the humidity of the soil air in the formation of sclerotia of *Rhizoctonia solani* Kühn was suggested but not supported by experimental evidence. According to the data obtained from Petri-plate culture in that study, it was concluded that most arable soils had sufficient phosphorus, potassium, calcium, and other mineral elements available for good growth of the pathogen. High concentrations of nitrogen and phosphorus in the nutrient agar appeared to favor the production of sclerotia, but it was not clear whether this effect was a nutrient response or a result of toxicity of the chemical to the fungus. The evidence from this and subsequent experiments appeared to place increasing emphasis on the humidity of the soil air and less on the importance of the mineral elements in soil. Also, as a result of various studies it was suspected that sclerotia would form rather freely in soil and on a variety of other materials, in addition to the surface of tubers. Finally, as Bisby, James, and Timonin (1) had shown that *Trichoderma lignorum* suppressed the growth of *R. solani* on nutrient agar, a study of its effect on sclerotia formation in soil was suggested. In the present paper, experimental data are presented on the formation of sclerotia in natural and steam-sterilized soil, on the effects of antagonism by *T. lignorum*, and on the importance of soil temperature.

METHODS.—The pathogen was increased in steam-sterilized black Edmonton loam soil. The soil used in the various units of the experiment was amended, steam sterilized, or natural, as required. Also, this soil was brought to the laboratory in its virgin state and found to be apparently free from *R. solani*. The soil containers were 1-liter Erlenmeyer flasks fitted with cotton plugs. Each flask contained approximately 900 g of soil. The water content of the soil was approximately optimum, or about 30 per cent of its moisture-holding capacity (m.h.c.), and that of the dry series was approximately 20 per cent of its m.h.c. when infested.

One part of soil-grown inoculum of *R. solani* was mixed with 9 parts soil. Where the experiment required that sterilized soil be recontaminated with natural soil, the inoculum of *R. solani*, natural soil (at the rate of 1 generous teaspoonful per flask), and the sterilized soil were mixed on a clean paper. This prepared soil was then put into flasks and incubated at approximately 61° and 75°F. To determine the number of sclerotia, the soil of the 4 flasks of each unit was mixed thoroughly in 1 pile; from it a 500-cc sample was removed and the sclerotia sifted out and counted.

EFFECT OF STEAM STERILIZATION OF NATURAL SOIL ON THE FORMATION OF SCLEROTIA.—The number of sclerotia that developed in sterilized soil recontaminated with natural soil was compared to that in natural soil amended with sodium nitrate at a rate known to favor vigorous hyphal growth of *R. solani*. Wet and dry units of each soil mixture were incubated for 30 days at 61° and at 75°F. In general the sclerotia were uniformly large.

More sclerotia developed at 61° than at 75°F in all soil treatments (Table 1).

In steam-sterilized soil under pure culture conditions, the hyphal growth of *R. solani* was vigorous, but only a few sclerotia developed. In striking contrast to these results, sclerotia developed abundantly when this steam-sterilized soil infested with *R. solani* was mixed with a small quantity of natural soil.

The number of sclerotia in the natural soil control was roughly 1/6 to 1/5 that in the recontaminated

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TABLE 1.—*Number of sclerotia in samples of natural soil, with and without added sodium nitrate, and of sterilized soil that were adjusted to different moisture levels, artificially infested with Rhizoctonia solani, and incubated for 30 days at different temperatures*

Soil water content (percentage of m.h.c.)	Temperature °F	Number of sclerotia in 500-cc sample[a]			
		Natural soil		Sterilized soil	Sterilized soil plus natural soil[c]
		No $NaNO_3$	$NaNO_3$[b]		
20	61	7	12	1	61
	75	5	3	0	26
30	61	12	10	5	67
	75	1	0	0	21

[a] Soil in 4 flasks was mixed, and sclerotia were counted in a 500-cc sample of this.
[b] 0.43 g per 100 g soil.
[c] One teaspoon per flask, which contained 900 g soil.

steam-sterilized soil. The addition of sodium nitrate to the natural soil infested with *R. solani* did not affect the number of sclerotia produced.

Approximately as many sclerotia formed in the dry soil as in the wet soil. This result suggests that the humidity of the soil air may have little or no effect on sclerotia formation; however, as both dry and wet soils were held in cotton-plugged flasks, air humidity in each container might have been the same (4).

EFFECT OF TRICHODERMA LIGNORUM ON SCLEROTIA FORMATION.—Bisby, James, and Timonin (1) demonstrated that *T. lignorum* suppressed the growth of *R. solani* on nutrient agar. Daines (2), however, was unable to control stem canker of potato by the use of that fungus.

Of a total of 7 soil series for this experiment, 4 were of steam-sterilized soil and 3 were of natural soil. All series received soil-grown inoculum of *R. solani*. One of the sterilized-soil series was recontaminated, as already described, with natural soil; another with natural soil and spores of *T. lignorum;* and another with *T. lignorum* and unsterilized cornmeal. The fourth series, which received only *R. solani*, served as a control. In the 3 natural-soil series, 1 was used for a control; another received cornmeal only; and the third, *T. lignorum*. Cornmeal was added at the rate of 15 g per flask. Natural soil and spores of *T. lignorum* were added at the rate of 1 teaspoonful per flask. The incubation period was 30 days and the temperature 61°F. The sclerotia found in 500 cc of representative soil samples of each series were counted.

In natural soil, the inoculum of *T. lignorum* did not depress sclerotia formation significantly; the number of sclerotia in the control series was 9, and that in the infested series, 7. The addition of cornmeal to natural soil favored abundant growth of *T. lignorum*, but no sclerotia developed.

No sclerotia formed when inoculum of *T. lignorum* was added to sterilized soil, whether or not it was recontaminated with natural soil. The addition of unsterilized cornmeal produced the same result. In contrast, the sterilized soil seeded lightly with natural soil yielded 84 sclerotia, whereas only 2 sclerotia were found in the sterilized soil control.

Trichoderma lignorum characteristically grew along the hyphae of *R. solani*, as if attracted by the latter (Fig. 1, B). Many such cultures incubated in artificially infested soil were examined, but actual penetration of a living cell of *R. solani* by *T. lignorum* was not observed. It is concluded, therefore, that the obvious antagonistic effect of *T. lignorum* results from outside contact and not from penetration of *R. solani*.

WHERE SCLEROTIA MAY FORM.—It is well known that sclerotia form freely on the surface of potato tubers. In the experiments with sterilized and natural soil (Table 1), sclerotia formed on the glass walls of the Erlenmeyer flasks. Apparently condensed moisture on these walls favored development of sclerotia.

In another experiment, various materials were added to flasks containing sterilized soil that was of optimum moisture content and that was recontaminated with 1 teaspoonful of natural soil per flask. The materials added included 1-in. lengths of clean glass tubing, varying from about $\frac{1}{8}$ to $\frac{1}{2}$ in. in diameter; 1-in. lengths of oat, wheat, barley, and brome grass straw; small stones; bits of wood; and bits of metal. These were mixed uniformly with the infested soil in the experimental flasks. The soil was incubated for 30 days at 61°F, then sclerotia formation on each material was observed.

Sclerotia formed readily on all the objects used in the test, especially in the inside of the cereal and

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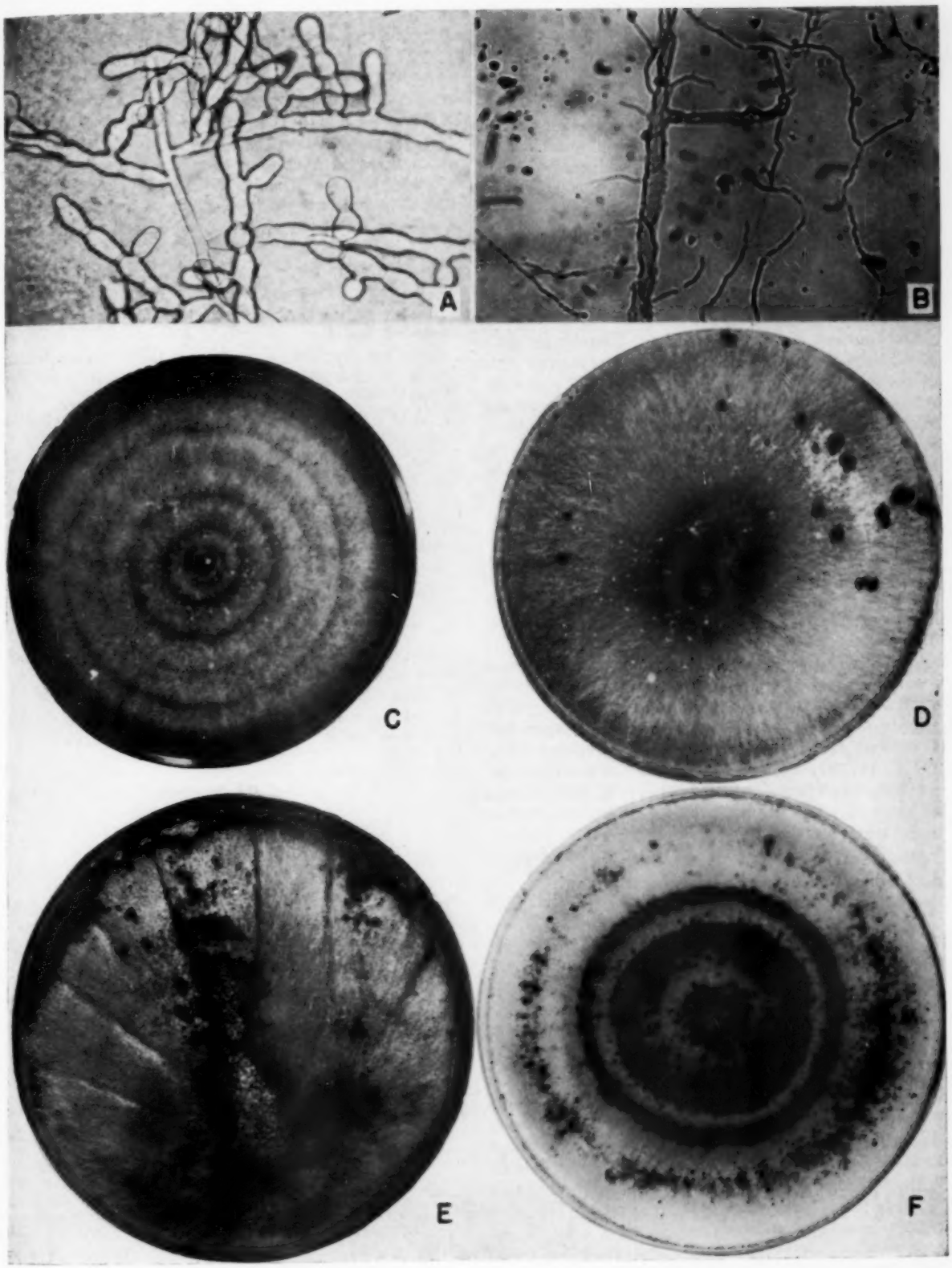
FIG. 1. Photographs of sclerotia formed by *Rhizoctonia solani*, including isolates that exhibit different capabilities to develop sclerotia at the same temperature on nutrient agar media. A) Early stage in growth of a sclerotium, showing barrel-type cells developing from a normal hyphal cell. B) Hyphae of *Trichoderma lignorum* adhering to surface of hyphae of *R. solani* in soil. C, F) Isolates with diurnal growth rings, C) without sclerotia, F) with abundant sclerotia. D, E) Isolates showing different patterns of sclerotia formation


A
B
C
D
E
F

grass stems and of the glass tubing. Large and small sclerotia formed on the surface of the pieces of stone and wood, particularly in crevices.

SCLEROTIA FORMATION ON SYNTHETIC AGAR.—The development of sclerotia in soil under field conditions is extremely irregular, but on nutrient agar, where moisture conditions are favorable, most isolates of *R. solani* usually produce sclerotia. This behavior suggests that the moisture factor may be of more importance in sclerotia formation than are soil nutrients or even the strain of the organism. If either the concentration of "staling products" or the activity of enzymes are important factors in the formation of sclerotia, it is conceivable that they would be more effective on nutrient agar than in soil, where they might be neutralized.

On nutrient agar, characteristic barrel-type cells of sclerotia always emerged from the rectangular cells of the normal hyphae (Fig. 1, A). Usually the development was from new hyphal growth, but sclerotial initials also arose from old living cells, either colored or hyaline. Normal hyphal cells emerged from the barrel-type cells of the surface of the sclerotium. In some isolates, the sclerotia became black sooner than they did in other isolates.

Although sclerotia tended to form first around the site of the original inoculum, mature sclerotia developed at random over the surface of the plate. On agar media, the sclerotial stimulation usually occurred in conjunction with new hyphal growth, but sometimes white cottony patches of immature sclerotia appeared later (Fig. 1, E). Often initiation and development of sclerotia seemed to be stimulated by some factor with which an obvious diurnal growth pattern was involved (Fig. 1, F). Other isolates had a similar pattern, yet no sclerotia developed (Fig. 1, C). This variability suggested that sclerotia formation depends upon a delicate balance of such factors as enzymes, toxic growth products and concentration of moisture.

DISCUSSION.—Adequate mineral elements for abundant production of sclerotia were present in the soils used. Consequently the data presented in this paper indicate that a number of factors, including temperature, antagonism, the genetic constitution of *R. solani*, and possibly the release of certain chemicals by associated fungi, are more important in sclerotia formation than are native mineral elements or plant material in normal soil.

The data in Table 1 suggest that it is not the total water content of the soil but rather the condensation of the moisture in the soil air on surfaces that is important for new hyphal growth and for the formation of sclerotia. If this be true, the widely recognized incidence of sclerotia on potato tubers that remain in the soil after the tops have withered in autumn may be correlated with favorable conditions for such condensation at that time.

The fact that *R. solani* grows so vigorously but produces relatively few sclerotia in steam-sterilized soil (Table 1) seems to be convincing evidence that normal nutrition is of secondary importance. The abundance of sclerotia in sterilized soil recontaminated with a relatively small quantity of natural soil suggests that there are other stimuli more important than temperature, nutrition, and moisture.

If the stimuli that initiate the barrel-type sclerotial cells from normal cells are of chemical nature and emanate from *R. solani*, they apparently are more effective on a nutrient agar than in sterilized soil. Also, if certain other associated fungi produce the necessary chemicals as well as or better than *R. solani* does, then the faster growth of these associated fungi than of other fungi present in the recontaminated sterilized soil might explain the stimulation of sclerotia formation obtained when natural soil was added to sterilized soil.

That the genotype of the fungus also is an important factor in sclerotial formation is indicated by the different capabilities of different isolates (Fig. 1, C, D, E, F) to produce sclerotia. This is supported by Houston (3).

For the inhibition of sclerotia formation by an antagonistic fungus to be effective, the growth of the antagonist must be abundant. For example, the liberal seeding of a natural soil with *T. lignorum* failed to inhibit formation of sclerotia, whereas a similar seeding either of natural soil amended with cornmeal or of sterilized soil reinfested with natural soil was very effective. Evidence from a previous study (7) showed that *T. lignorum* developed abundantly in a sterilized soil but poorly in a natural unamended soil. On the basis of these results it seems doubtful if sclerotia formation in field culture would be reduced noticeably by antagonism from associated fungi, although the persistence of *R. solani* might be affected (5, 6, 7).

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RELATIONSHIP OF NEMATODES TO THE DEVELOPMENT OF FUSARIUM WILT IN COTTON [1]

W. J. Martin, L. D. Newsom, and Jack E. Jones [2]

SUMMARY

Development of Fusarium wilt was studied in cotton growing in steam-sterilized soil artificially infested with populations of nematodes alone, with *Fusarium oxysporum* f. *vasinfectum* alone, and with combinations of each nematode population and the Fusarium. Pure populations of *Meloidogyne incognita*, *M. incognita acrita*, *Trichodorus* sp., *Tylenchorhynchus* sp., and *Helicotylenchus* sp. were used. The nematodes reproduced abundantly on cotton varieties Deltapine 15 (wilt-susceptible) and Coker 100 Wilt (wilt-resistant). Of the 5 nematodes used in the tests, only *Meloidogyne incognita* and *M. incognita acrita* significantly increased incidence of wilt in the 2 varieties. There appeared to be differences among isolates of Meloidogyne in their ability to increase incidence of wilt. Severe injury to the cotton was recorded as a result of infestations of Meloidogyne only. Little or no injury to cotton was caused by the other 3 genera.

That there is an association of nematodes with severe wilt in cotton was suggested with Atkinson's observations made as early as 1892 (1). More recently Barker, Taylor, Smith, and others (2, 8, 9, 11, 12) have contributed to our understanding of this association. Then in 1948 Smith (10) reported that cotton wilt, as well as nematodes, was controlled by soil fumigation with ethylene dibromide, which was conceded generally as having little if any fungicidal action. Since 1950, experiments in Louisiana on fumigation of soils infested with the cotton wilt organism also resulted in striking control of wilt (7). In these experiments, the species of nematodes found in fumigated and nonfumigated plots were of the following plant parasitic genera: *Meloidogyne*, *Trichodorus*, *Tylenchorhynchus*, *Helicotylenchus*, and *Pratylenchus*. To determine which of these nematodes were involved in the wilt-nematode complex, it was necessary to obtain relatively pure populations of each species. This was done, except with *Pratylenchus* sp.,[3] by hand-picking individuals of each species and allowing them to propagate on suitable host plants growing in steam-sterilized soil contained in clay pots (4).

Meanwhile Holdeman and Graham (3) reported that the sting nematode, *Belonolaimus gracilis* Steiner, 1949, enhanced the development of Fusarium wilt in both susceptible and resistant cotton plants. Since the sting nematode had not been recorded in Louisiana soils infested with the wilt organism, it seemed of interest to determine the role in the complex of each of the nematode species that had been propagated in pure populations for that purpose.

This paper gives results obtained from a study of the role in the wilt-nematode complex of the following nematodes: *Meloidogyne incognita* (Kofoid & White, 1919) Chitwood, 1949; *Meloidogyne incognita acrita* Chitwood, 1949; *Trichodorus* sp., *Tylenchorhynchus* sp., and *Helicotylenchus* sp.[4] A brief abstract of these results has been published (6).

MATERIALS AND METHODS.—The effects of the nematodes alone or in combination with *Fusarium oxysporum* Schlecht. f. *vasinfectum* (Atk.) Snyd. & Hans. were studied on 2 varieties of cotton (*Gossypium hirsutum* L.); namely, Deltapine 15, a wilt-susceptible variety widely planted in Louisiana, and Coker 100 Wilt, a wilt-resistant variety frequently planted in infested soils.

Each experiment consisted of the following 4 treatments: 1) check, 2) nematodes only added, 3) Fusarium only added, and 4) both nematodes and Fusarium added. Each treatment was replicated 4–6 times in steam-sterilized soil in 8-in. pots. The pots were arranged in a randomized block design on a greenhouse bench. Greenhouse temperatures varied somewhat, but attempts were made to maintain 80°F.

The populations of nematodes were added in water suspensions, usually in numbers as great or greater than that normally occurring in heavily infested soils. Each pot of soil received an equal volume (usually 25 ml) of a thoroughly agitated suspension. The Fusarium in the root-knot tests was added as a finely chopped mycelium and spore suspension from 10-day-old cultures grown on Czapek's solution. Each pot of

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[3] Several earlier attempts to propagate hand-picked specimens of *Pratylenchus* sp. (Louisiana collections Nos. 1, 6, 28, 128) and *Pratylenchus brachyurus* Godfrey (Louisiana collection No. 27) on corn and cotton plants were not successful. Recently, *Pratylenchus* sp. was successfully propagated on cotton, and controlled experiments are underway with that organism.

[4] Since nematologists seemed in agreement that 3 of the species used in this study were undescribed forms, large numbers of each species were preserved in 5 per cent formaldehyde after killing with gentle heat. These specimens were placed in the Louisiana Nematode collection in the Department of Plant Pathology, Louisiana State University, as the following numbers: *Meloidogyne incognita* (Louisiana No. 282 and 347), *M. incognita acrita* (Louisiana No. 308), *Trichodorus* sp. (Louisiana No. 5 and 335), *Tylenchorhynchus* sp. (Louisiana No. 42, 92, 171, and 333), and *Helicotylenchus* sp. (Louisiana No. 4, 12, 93, and 170).

soil to be infested received 50 ml of the Fusarium suspension, whereas the noninoculated controls received 50 ml of sterilized Czapek's solution. Three populations of root-knot nematodes were used, each originating from single-egg-mass isolates propagated on roots of Marglobe tomato (*Lycopersicon esculentum* Mill.) growing in pots of steam-sterilized soil in the greenhouse. Two of these populations (Louisiana No. 282 and 347) were identified as *Meloidogyne incognita*, and the third (Louisiana No. 308) was *M. incognita acrita*. Each pot of soil to be infested received the equivalent of 50 mature egg-masses from tomato roots as described above.

In the Trichodorus, Helicotylenchus, and Tylenchorhynchus tests, the Fusarium inoculum was added as 15-day-old cultures on sterilized oats. In each pot of soil to be infested, 50 g of the oat inoculum were added, and 50 g of sterilized oats were added to the controls and to soil containing only nematodes. The oats were thoroughly mixed with the top 4 in. of soil in each pot. The nematode suspensions and wilt inoculum were introduced into the soil at the time of seeding by pouring the suspensions directly over the acid-delinted seeds before they were covered with soil. Twenty seeds were planted in each pot.

Data on wilt were taken at least twice weekly during the course of the test. Plants were tagged as wilt-affected when they exhibited leaf symptoms of tipburn. In doubtful cases, the petioles were examined for vascular discoloration. Otherwise, wilt-affected plants were allowed to remain in the pots throughout the course of the experiment. When the experiments were ended, data were taken on vascular discoloration of stems at the crown of each plant.

RESULTS.—*Root-knot nematodes.*—Two experiments were made with variety Deltapine 15 and *M. incognita* (Louisiana No. 282). In the first, there were 4 replications, and notes were recorded over a period of 42 days. The mean percentage of wilt-affected plants, based on external symptoms only, for the 4 treatments in this test were as follows: controls, 0.0; *M. incognita* alone, 4.2; Fusarium alone, 39.4; and *M. incognita* + Fusarium, 97.9. Severe root knot had developed on all plants in the nematode-infested pots.

In the second experiment with Deltapine 15, detailed data were obtained over a longer period of time (Table 1). The percentage of wilt-affected plants was increased greatly in the presence of the root-knot nematodes. Some wilt developed in plants growing in soil that received only root-knot nematodes, presumably due to seed-borne infection. Reduction in green weight was striking where both root knot and Fusarium were present.

In similar tests with the variety Coker 100 Wilt, severe root knot developed again on all plants in nematode-infested soil (Table 2). The percentage of wilt-affected plants in this wilt-resistant variety was comparatively small where Fusarium only was used. There was, however, a strikingly higher percentage of wilt-affected plants in the 3 treatments where nematodes were added to the Fusarium inoculum. These results clearly indicate differences among the 3 root-knot populations in their ability to render the Coker 100 Wilt variety more susceptible to Fusarium wilt. *M. incognita* (Louisiana No. 282) was the most effective in increasing the amount of wilt, and *M. incognita acrita* was the least effective. These results were not unexpected, however, in view of the recent report of wide variation in adaptability and pathogenicity among root-knot nematodes on cotton (5).

The reduction in green weight of plants in the nematode-infested treatments was quite striking (Table 2). Such severe damage to cotton plants by root-

TABLE 1.—*Mean[a] percentage of wilt-affected plants of the wilt-susceptible variety of cotton (Deltapine 15) and green weight of plants growing in sterilized soil infested with Meloidogyne incognita, with Fusarium oxysporum f. vasinfectum, or with a combination of the 2 organisms*

Treatment	Percentage wilt-affected plants: After 82 days	Percentage wilt-affected plants: After 134 days	Green wt. of plant after 157 days	Mean no. nematodes per pot at end of test
			g	
Check	0.0	0.0	189.9	0
Fusarium	4.2	26.6	187.7	0
M. incognita	4.2	35.0	145.5	1005
M. incognita + Fusarium	65.8	100.0	48.5	87
LSD 5%	12.49	25.42	33.82	434
1%	17.27	35.16	46.78	600

[a] Mean of 6 replicates.

TABLE 2. *Mean[a] percentage of wilt-affected plants of a wilt-resistant variety of cotton (Coker 100 Wilt) 35, 55, and 104 days after planting in soil infested with 3 different isolates of root-knot nematodes, each alone and in combination with Fusarium oxysporum f. vasinfectum, and mean percentage of plants alive and green top weights of plants 104 days after planting*

Treatment	Percentage of wilt-affected plants[b] after: 35 days	55 days	104 days	Percentage of plants alive	Green wt.
					g
Check	0.0	0.0	0.0	99.0	85.0
Fusarium	3.0	3.9	6.8	94.4	86.0
M. incognita acrita (La. No. 308)	0.0	0.0	0.0	99.0	40.8
M. incognita (La. No. 347)	0.0	0.0	0.0	86.0	37.6
M. incognita (La. No. 282)	0.0	0.0	0.0	85.2	35.6
La. No. 308 + Fusarium	22.9	51.0	66.2	37.6	25.2
La. No. 347 + Fusarium	24.5	62.8	74.4	25.8	18.2
La. No. 282 + Fusarium	43.6	68.3	83.0	23.2	17.8
LSD 5%	8.30	11.92	11.90	14.87	11.43
1%	11.14	15.99	15.96	19.95	15.34

[a] Mean of 5 replicates.
[b] Percentage of wilt-affected plants after 35 and 55 days was based on external symptoms only; that after 104 days included vascular discoloration.

TABLE 3.—*Mean[a] percentage of wilt-affected cotton plants and green weight of plants growing in sterilized soil infested with Trichodorus sp., Fusarium oxysporum f. vasinfectum, or a combination of the 2 organisms*

Host variety and treatment	Percentage wilt-affected plants: External symptoms only	Percentage wilt-affected plants: Including vascular discoloration	Green wt.	No. nematodes per pot at end of test
			g	
Deltapine 15[b]				
Check	11.7	18.9	44.0	0
Trichodorus	1.8	20.5	29.2	10,640
Fusarium	49.5	87.2	48.2	0
Trichodorus + Fusarium	47.9	83.1	48.4	700
LSD 5%	16.68	23.24	14.53	
1%	22.82	31.80	N.S.	
Coker 100 Wilt[c]				
Check	0.0	2.9	74.6	0
Trichodorus	2.9	2.9	71.0	6,320
Fusarium	28.6	48.8	90.8	0
Trichodorus + Fusarium	12.9	46.9	95.0	9,460
LSD 5%	20.36	20.92	14.55	
1%	27.86	28.63	N.S.	

[a] Mean of 5 replicates.
[b] At the beginning of the test there were 6320 nematodes per pot in the Trichodorus series and 9460 in the Trichodorus + Fusarium series. Since this experiment was planted in the same pots used in the experiment with the Coker 100 Wilt variety, the mean number of nematodes per pot at the start of this test was the same as the mean number of nematodes per pot at the end of the experiment with Coker 100 Wilt. Final data were taken in this test 140 days after planting.
[c] At the beginning of this test there were 250 nematodes per pot in both the Trichodorus (alone) and in the Trichodorus + Fusarium series.

TABLE 4.—*Mean[a] percentage of wilt-affected cotton plants and green weight of plants growing in sterilized soil infested with Helicotylenchus sp., Fusarium oxysporum f. vasinfectum, or a combination of the 2 organisms*

Host variety and treatment	Percentage wilt-affected plants: External symptoms only	Percentage wilt-affected plants: Including vascular discoloration	Green wt.	No. nematodes per pot at end of test
			g	
Deltapine 15[b]				
Check	0.0			
Helicotylenchus	0.0			
Fusarium	66.8			
Helicotylenchus + Fusarium	46.8			
LSD 5%	18.70			
1%	25.90			
Coker 100 Wilt (Test 1)[c]				
Check	0.0	0.0	45.6	0
Helicotylenchus	2.5	12.5	53.8	9,000
Fusarium	14.1	29.8	67.0	0
Helicotylenchus + Fusarium	11.6	21.1	64.6	7,360
LSD 5%	9.18	N.S.	14.46	
1%	N.S.	N.S.	N.S.	
Coker 100 Wilt (Test 2)[d]				
Check	2.0	6.9	28.8	200[e]
Helicotylenchus	0.0	0.0	24.4	131,180
Fusarium	23.4	61.3	47.0	0
Helicotylenchus + Fusarium	34.7	75.2	40.4	100,240
LSD 5%	16.07	27.96	10.70	
1%	21.99	38.27	14.64	

[a] Mean of 5 replicates.
[b] At the beginning of the test there were 2000 nematodes per pot. Final data on this test were taken 53 days after planting.
[c] At the beginning of this test there were 3000 nematodes per pot in both the Helicotylenchus series and the Helicotylenchus + Fusarium series. Final data were taken 97 days after planting.
[d] This test was made in the same pots of soil used in above test 1. Therefore, at the beginning of the test there were 9000 nematodes per pot in the Helicotylenchus series and 7360 nematodes per pot in the Helicotylenchus + Fusarium series. Final data were taken in this test 140 days after planting.
[e] Two of the check pots contained nematodes at the end of this test, one containing 800 nematodes and one 200. These apparently were introduced accidentally during the course of the test.

TABLE 5.—*Mean[a] percentage of wilt-affected cotton plants and green weight of plants growing in sterilized soil infested with Tylenchorhynchus sp., Fusarium oxysporum f. vasinfectum, or a combination of the 2 organisms*

Host variety and treatment	Percentage wilt-affected plants: External symptoms only	Percentage wilt-affected plants: Including vascular discoloration	Green wt.	No. nematodes per pot at end of test
			g	
Deltapine 15[b]				
Check	0.0	3.8	38.8	0
Tylenchorhynchus	0.0	0.0	30.6	26,600
Fusarium	35.2	58.7	46.4	0
Tylenchorhynchus + Fusarium	40.1	63.2	37.0	10,380
LSD 5%	19.65	25.87	9.72	
1%	26.88	35.41	N.S.	
Coker 100 Wilt[c]				
Check	0.0	0.0	54.4	0
Tylenchorhynchus	9.0	9.0	59.4	2,160
Fusarium	18.8	43.5	88.6	0
Tylenchorhynchus + Fusarium	13.6	43.6	89.0	7,560
LSD 5%	N.S.	22.34	8.07	
1%	N.S.	30.57	11.04	

[a] Mean of 5 replicates.

[b] At the beginning of this test there were 2160 nematodes per pot in the Tylenchorhynchus series and 7560 nematodes per pot in Tylenchorhynchus + Fusarium series. Since this experiment was planted in the same pots used in the experiment with the Coker 100 Wilt variety, the mean number of nematodes per pot at the start of this test was the same as the mean number of nematodes per pot at the end of the experiment with Coker 100 Wilt. Final data were taken 140 days after planting.

[c] At the beginning of this test there were 1800 nematodes per pot in both the Tylenchorhynchus series and the Tylenchorhynchus + Fusarium series. Final data on this test were taken 97 days after planting.

knot nematodes alone was somewhat unexpected. Reduction in green weight was greater, however, where both nematodes and Fusarium were present.

Other species.—*Trichodorus* sp. did not increase the incidence of disease in either Deltapine 15 or Coker 100 Wilt (Table 3). On Deltapine 15, there appeared to be a decrease in green weight where Trichodorus only was present but not where Trichodorus + Fusarium was present. On Coker 100 Wilt, the 2 sets of plants that received Fusarium inoculum were significantly greater in green weight than the controls or those receiving only Trichodorus. Apparently this was due to the difference caused by the action of Fusarium on the sterilized oats, better growth being obtained in all experiments where oats + Fusarium were added than where sterilized oats only were added.

Helicotylenchus sp. did not increase incidence of wilt in either Deltapine 15 or Coker 100 Wilt (Table 4). In the tests on Coker 100 Wilt, the 2 sets of plants receiving Fusarium inoculum were higher in green weight than the controls or those receiving only Helicotylenchus.

Tylenchorhynchus sp. did not increase wilt in either Deltapine 15 or Coker 100 Wilt (Table 5).

DISCUSSION.—The results reported herein clearly indicate that *Meloidogyne incognita* and *M. incognita acrita* are the nematodes mainly responsible for causing increases in the incidence of cotton wilt in Louisiana soils. With the exception of *Pratylenchus* spp., which were not studied here, the other plant parasitic species commonly occurring in areas where cotton-wilt is prevalent in Louisiana do not appear to be capable of increasing incidence of wilt in cotton. In our studies, *Trichodorus* sp., *Tylenchorhynchus* sp., and *Helicotylenchus* sp. all increased to large populations in sterilized soil planted to cotton, but there was no indication of any increase in the incidence of wilt in their presence. Also, there was little or no indication of injury to the plants growing in soil infested with these large populations of nematodes. On the contrary, there was severe injury to the plants growing in the soil infested with only Meloidogyne. The apparent difference among isolates of Meloidogyne in ability to increase incidence of Fusarium wilt in cotton might prove valuable in studies on the nature of this relationship between nematode infestation and the development of Fusarium wilt in cotton.

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HISTOLOGIC EXPRESSION OF SUSCEPTIBILITY AND RESISTANCE IN BARLEY TO STRAINS OF HELMINTHOSPORIUM GRAMINEUM [1]

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SUMMARY

Histological studies were made of the reaction of 2 strains of *Helminthosporium gramineum* Rab. on 4 barley varieties varying in resistance to the organism. When the variety was susceptible to the fungus strain, the fungus penetrated the coleorhiza by means of appressoria and hyphal pegs. It advanced intracellularly through the parenchymatous regions and usually intercellularly through meristematic areas. After the mycelium became established in the entire length of the seedling culm, rapid elongation of the seedling resulted in the establishment of isolated loci of infection in the internodes. Spread of mycelium in the host also occurred through the lumina of xylem vessels. In the resistant varieties penetration by the fungus was similar to that described above; however, a host-parasite interaction occurred below the outer 2 or 3 layers of host cells. What appeared to be a form of hypersensitive reaction resulted in the formation of necrotic pockets in the coleorhiza tissue. Mycelium was inactivated in these regions and the plant proceeded to develop normally.

Barley stripe, incited by *Helminthosporium gramineum* Rab., occurs in most barley areas. The disease has been studied by other workers with respect to varietal reaction, inheritance of resistance, and fungus variability. Results on the histology of the susceptible reaction are controversial, however, and the resistant reaction has not been reported.

Ravn (4) concluded that *H. gramineum*, like certain of the smut fungi, established itself in the growing point and then spread to each young part of the plant as that part differentiated. Smith (6) described penetration occurring through the coleoptile by means of an appressorium and a hyphal peg. The leaves were then invaded successively as the fungus grew inward, and the stem apex was attacked only in the final phases of the disease. A few years later, Stelzner (7) reported penetration occurring through the coleorhiza and the seminal roots, with invasion of the leaves taking place through the xylem vessels.

MATERIALS AND METHODS.—Two strains of *H. gramineum*, designated as C-1 and GHA2, were used by Shands and Arny (5) and Arny (1), respectively. Both strains had been carried in culture on potato-dextrose agar by mass transfers. They had been stable in cultural characters and in pathogenicity for over 10 years.

Four barley varieties were used in the present studies. Atlas (C.I. 4118) was highly susceptible to strain GHA2 and highly resistant to strain C-1. Oderbrucker (C.I. 4666) gave the reciprocal reactions in that it was highly susceptible to C-1 and highly resistant to GHA2. Colsess (C.I. 5979) was highly susceptible to both strains, whereas selection B39-55 (from a cross of Atlas and Oderbrucker) was highly resistant to both strains.

The inoculation technique as described by Arny and Shands (2) was used. The fungus was cultured on moistened, autoclaved wheat kernels. Barley seedlings became infected during germination in this fungus inoculum. For histological studies the barley material was fixed at various stages in formalin-acetic-alcohol and dehydrated in a tertiary-butyl-alcohol series (3). The sectioned material (10 μ usually) was stained with Conant's quadruple stain (3).

PATHOLOGICAL HISTOLOGY. — *Susceptible reaction.*— In varieties that were susceptible to specific strains of the fungus, penetration occurred through the coleorhiza (Fig. 1) under the artificial inoculation conditions. Appressoria formed on the epidermal surface beneath the gelatinous protective covering of the coleorhiza.

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Hyphal pegs penetrated either into or between the epidermal cells. A depression in the cell wall at the point of penetration indicated that mechanical pressure was exerted during penetration. A slight host reaction was indicated at this stage by a transitory red-staining area just ahead of the slightly swollen hyphal tip.

In the coleorhiza, unbranched hyphal strands proceeded intracellularly and usually parallel to the longi-

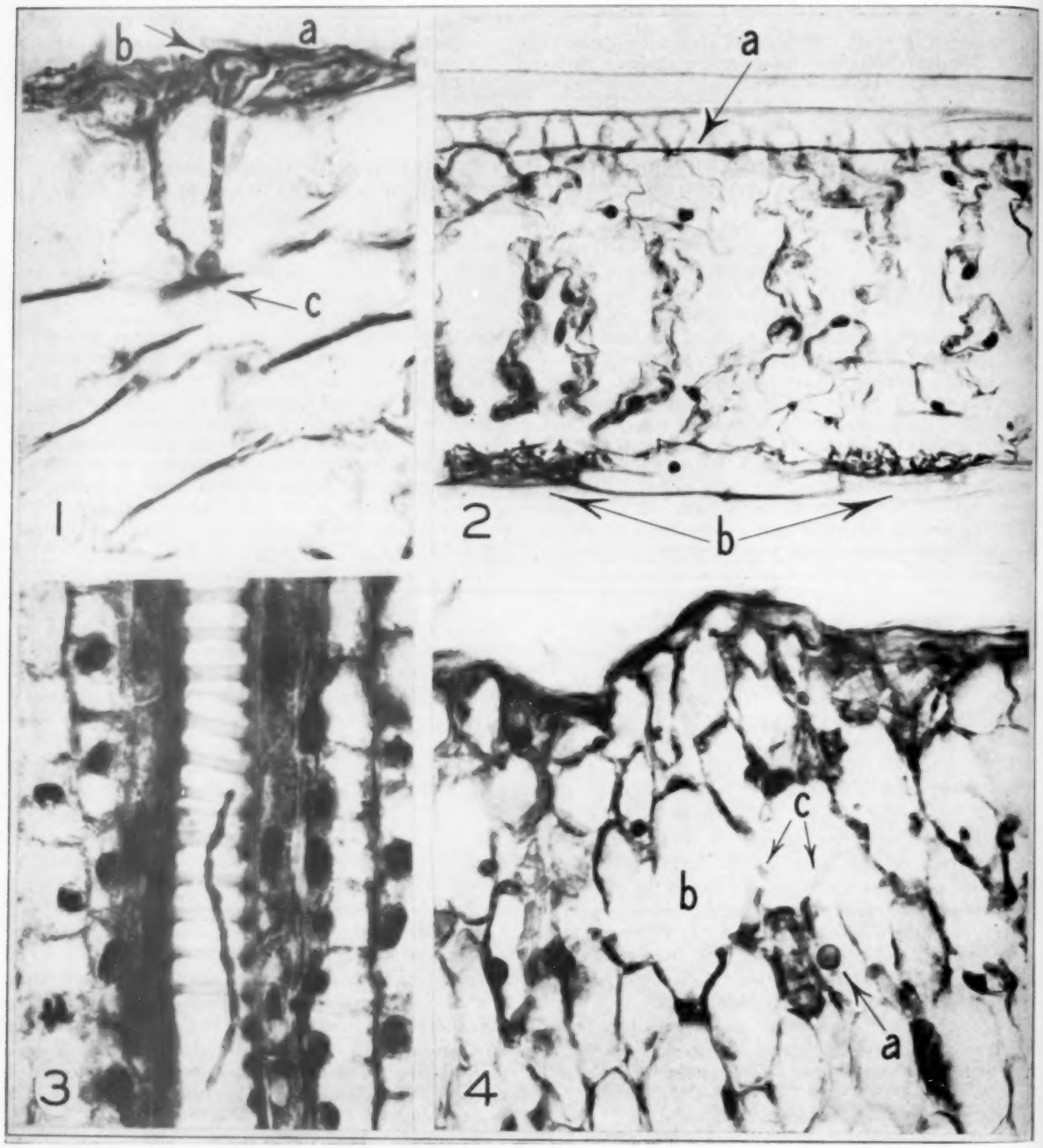

FIG. 1–4.—Fig. 1. Intercellular penetration into coleorhiza of Oderbrucker by strain C-1 (susceptible reaction) 5 days after inoculation. a) Protective layer over coleorhiza. b) Appressorium. c) Temporary reaction in area of hyphal tip.—Fig. 2. Longitudinal section through nearly mature leaf of Oderbrucker infected with strain C-1. a) An unbranched hyphal strand passing through apparently normal mesophyll cells. b) Aggregations of mycelium in substomatal cavities.—Fig. 3. Longitudinal section through coleoptile of a 10-day-old Atlas seedling, showing hyphal strand of strain GHA2 in xylem vessel (susceptible reaction).—Fig. 4. Penetration of coleorhiza of 6-day-old Oderbrucker seedling by strain GHA2, showing advanced stage of resistant reaction. a) Dense, deeply staining host nucleus. b) Cavity formed by disintegrating host cells. c) Degenerate hyphal fragments. (Photographs made by Eugene Herrling.)

tudinal axis of the seedling. A continuous layer of parenchymatous cells joining the coleorhiza and the coleoptile allowed the fungus to progress rapidly upward to establish itself at the base of the leaves.

During the early stages of germination of the host, mycelial invasion of the newly formed leaves occurred only after they had reached a certain stage of physiologic maturity. Characteristically, the hyphae were fairly well advanced in the first true leaf, just starting invasion in the second leaf, but still at the base of the third leaf. This sequence existed when the mycelium was present along the entire base of the embryonic leaves.

In the young leaf the hyphae proceeded through the spongy mesophyll parallel to the vascular bundles (Fig. 2). The bundle sheaths served as structural barriers to the lateral spread of the mycelium. Growth through the mesophyll tissue was about equally intercellular and intracellular, even in regions where the spaces between the cells were large.

The host showed no staining reaction in the initial stages of leaf invasion by the fungus. Later the chloroplasts lost their ability to stain dark green and finally disintegrated into formless masses. Macroscopically, the changes in the chloroplasts were expressed in the leaf blade and sheath as characteristic pale yellow stripes that paled to white and finally turned brown.

In leaves approaching maturity, the mycelium assumed an entirely different habit of growth. The mycelium branched progressively, and in the later stages of invasion it ramified throughout the spongy mesophyll. Mycelial strands first aggregated into dense masses in substomatal cavities (Fig. 2) and later invaded the epidermal cells. The mesophyll cells eventually collapsed and disintegrated. Following the complete establishment of the mycelium in the spongy mesophyll, numerous strands of hyphae emerged through the stomata and epidermal cells. These hyphal strands developed into stout, dark-colored conidiophores protruding from the leaf surface. Conidia were produced on diseased plants during the time that disease-free barley plants were flowering.

From the coleorhiza, thin strands of mycelium developed slowly through the scutellar and coleoptilar nodes to establish themselves in the embryonic region of the primary culm. In this region the predominantly intercellular growth appeared to be slow, either because of the dense mass of rapidly dividing host cells or because of unfavorable physiologic conditions. The mycelial strands were tortuous, but their growth was directed toward the stem apex. The stem apex was not invaded at this early stage.

Approximately 3 weeks following germination, the internode between the scutellar and coleoptilar nodes and that between the coleoptilar and lower crown nodes elongated rapidly. As a result of this accelerated elongation the hyphal strands were broken, and isolated fragments of hyphae were found frequently in the lower internodes. Thus the host carried the infection loci upward in the various elongating tissues, and the hyphae, once infection was established, were immediately below the growing point of the primary culm. The stem apex was invaded only just before the spike emerged from the boot. The presence of the isolated loci of hyphae in the region of cellular differentiation and growth was established by a detailed study of serial sections of these tissues.

Hyphal advance in the tissues of the leaves and the culm also occurred by way of the lumina of the xylem vessels. This method of spread occurred more commonly in the leaves than in the other plant parts (Fig. 3). The initial penetration into the vessels was not observed, but the development of hyphae within them was generally evident. Once inside the vessel, the fungus was able to advance faster than it could through or between the host parenchyma cells.

In older tissues of the host, most of the xylem vessels contained various amounts of mycelium, and many of them were plugged mechanically. Growth outwardly from these vessels occurred only after disintegration of the walls. The few instances of leaf stripes appearing in isolated points well in advance of the general striped area possibly were associated with the rapidly advancing hyphae in the xylem vessels and local collapse of the vascular tissues in such areas.

Isolated areas showing a pathological reaction in the culm, as indicated by deep staining with safranin, were found in parenchymatous tissues adjacent to infected vessels. Mycelium was not evident in the parenchyma in these local reaction areas.

The seminal roots were invaded readily by *H. gramineum*. The intracellular mycelium ramified rapidly in the cortical tissues. This tissue soon became necrotic and finally disintegrated completely. The stelar cells resisted invasion for some time, but eventually succumbed to the fungus. Hyphae were found only rarely in the xylem vessels of the roots, and advance of the hyphae into other parts of the seedling occurred infrequently.

Resistant reaction.—The fungus penetrated the resistant barley seedlings through the coleorhiza by means of appressoria and hyphal pegs. The invading hyphae appeared to be compatible with the host through at least 2 layers of cortical cells.

The first indication of host-fungus incompatibility was a faint red staining of the intercellular regions immediately in advance of the hyphal tips. This staining reaction later was intensified to a deep red. Protoplasts enclosed within the host cell walls appeared to remain functional for some time. Later, as fungal development progressed, the host cytoplasm became granular and eventually disorganized with the collapse of cells.

The host nuclei condensed into spherical masses that stained a deep red. These nuclei frequently were dissociated from the surrounding cytoplasm, which also aggregated into irregular masses (Fig. 4).

In this manner a definite reaction zone was estab-

lished at the points of hyphal invasion of the resistant seedling. Mycelial strands were not observed beyond this zone, and those at the edges showed chemical change and reaction by failure to retain safranin. Thus both the host and parasite cells died in the invasion area, thereby preventing further disease development.

Penetration and development of mycelium in the roots of resistant varieties appeared to be the same as in susceptible varieties.

Discussion.—The observation that penetration into the barley seedling occurred through the coleorhiza and not through the coleoptile confirmed the findings of Stelzner (7); however, he considered root invasion by the fungus to be as effective in systemic development of the stripe disease as was penetration through the coleorhiza. The present investigation showed that whereas the roots were damaged considerably by the fungus, advance of mycelium from this source into other parts of the seedling occurred only rarely. Also the roots of resistant as well as susceptible barley varieties were attacked under the conditions of inoculation employed. This indicated that the invasion of seminal roots was independent of the resistant reaction. Penetration through the coleoptile, as reported by Smith (6), was not confirmed by Stelzner (7) nor by the present study.

The mycelium advanced intracellularly in parenchymatous tissue and intercellularly in active meristematic tissue. The path of the mycelium was mostly parallel to the long axis of the seedlings, but in disintegrated susceptible host tissue the mycelium ramified in all directions.

Stelzner (7) noted the extension of the fungus through the lumina of xylem vessels of the leaves and culms. He considered that invasion of the leaves occurred only by this method and that the spongy mesophyll was attacked only after the xylem walls disintegrated. The present study confirmed the development of mycelium in the vessels, but this was not considered to be the major means of leaf invasion. Mycelium was followed from the seedling culm into the spongy mesophyll and was often found in this tissue, even when the adjacent xylem vessels were not invaded. Smith (6) also found the mycelium to be mainly in the mesophyll, and considered that the vascular bundles limited lateral spread.

The establishment of fragments of mycelium in the internodes as a result of rapid elongation of the seedling was observed also by Stelzner (7). In this manner isolated loci of infection were initiated in each internode, and it was not necessary for the fungus to grow through the older nodes in order to reach younger leaves and finally the growing point.

Resistance to barley stripe was physiologic in nature and appeared to be a form of hypersensitivity despite the fact that the fungus will grow well as a saprophyte. In contrast to the susceptible reaction, in which the fungus ramified throughout the collapsed host tissues, the mycelium died in the reactive zones of resistant varieties. Damage to the resistant host was limited, therefore, to isolated spots in the coleorhiza and did not affect normal development of the barley seedling.

It remains to be established whether the observations made in the present study under artificial inoculation conditions will hold under conditions of natural infection.

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A SAND-CULTURE TECHNIQUE FOR THE ISOLATION OF FUNGI ASSOCIATED WITH ROOTS[1]

Stephen Wilhelm

SUMMARY

After incubation in moist sterile sand, washed surface-disinfested roots were examined for resting or reproductive structures of fungi, which were then dissected or trapped out for further culturing or study. The most typical root fungus flora on the hairy nightshade, *Solanum sarachoides*, growing as a weed in strawberry fields of central coastal California, consisted of a number of pathogens better known on other hosts. Among these were *Verticillium albo-atrum*, pathogenic to strawberry; *Colletotrichum atramentarium* and *Pyrenochaeta terrestris*, pathogenic to onion and strawberry; *Pythium ultimum*, pathogenic to tomato; *Macrophomina phaseoli*; *Fusarium roseum*, a species of Phoma; and a sterile fungus pathogenic to strawberry.

When it was found that the nightshade weed *Solanum sarachoides* Sendt. was a frequent symptomless carrier of *Verticillium albo-atrum* Reinke & Berth. (6) and that infection in a young plant was confined mainly to a certain few roots, ways of disclosing such infection were explored. The technique finally devised has proved useful not only for finding rootlet infection by Verticillium but also for revealing the presence of other root-invading (3) or root-surface (rhizoplane) fungi (1), especially of those producing resting structures. The technique has a great advantage over conventional culturing in that large quantities of roots, such as the nearly entire root system of a small plant, can be easily surveyed for root-invading fungi. It has not helped, however, in the isolation of Phycomycetous mycorrhizal fungi of the Rhizophagus type.

The method consists simply of burying a considerable quantity of previously washed and surface-disinfested roots in Petri dishes 2-in. deep containing coarse sterile moist sand. Roots should be active, glistening white, and without secondary tissues that become dark colored. Immersion in 0.1 per cent mercuric chloride for 1.5–2 minutes followed by 2 rinsings in sterile distilled water is an adequate disinfestation. Ordinary Petri dish tops containing a layer of 2 per cent water agar are used as covers and prevent both contamination and loss of moisture from the sand. Roots are removed to dishes of sterile water after 2, 3, or even 4 weeks' incubation in the sand and examined under the dissecting microscope. Resting or reproductive structures of fungi may be dissected out and mounted for more critical examination or transferred to culture media. Single spores often may be transferred from mature pycnidia, which release spores when the root containing them is placed in water. If the previous disinfestation was adequate, usually only slight disintegration of the roots takes place even after 4 weeks' incubation. Certain fast-growing fungi may be trapped out of the sand in a few days by placing sterilized seeds or bits of plant materials at short distances from the roots.

Nightshade roots that were glistening white when placed in the sand may have, upon removal, fruiting or resting bodies of several kinds of fungi. Aside from microsclerotia of *V. albo-atrum*, which form in the cortical and stelar areas of the root (Fig. 1, D), resting bodies of a gray sterile fungus commonly appear. In size and shape they conform largely to the invaded cortical cells (Fig. 1, C). A possible spore stage of infrequent occurrence, resembling Pyrenochaeta, has been reported for this fungus (7). The incubated roots may bear sclerotia of *Colletotrichum atramentarium* (Berk. & Br.) Taub., which in more favorable culture media develop into setose acervuli (Fig. 1, A), or they may be ruptured by the large spherical sclerotia of *Macrophomina phaseoli* (Maubl.) Ashby. They may bear pycnidia of a Phoma (Fig. 1, B), which in culture gives a dark rank-growing colony. Individual rootlets often become pink in the sand because of an invasion by *Pyrenochaeta terrestris* (Hansen) Gorenz, J. C. Walker, & Larson and may bear the small resting bodies of this fungus (Fig. 1, E). On the root surfaces, hyphal strands of *Rhizoctonia solani* Kühn, chlamydospore clumps of *Fusarium roseum* (Lk.) Snyd. & Hans. (Gibbosum type), and oospores of *Pythium ultimum* Trow may develop. These 9 different fungi were the commonly found root-surface or root-invading fungi found on *Solanum sarachoides* growing in strawberry fields in parts of central coastal California. Occasionally, a Sclerotinia forming extensive sclerotial sheets throughout the roots was encountered, as well as spores of species of *Curvularia*, *Dendryphium*, and *Stemphylium*.

In inoculation tests, some of these fungi, apparently innocuous to nightshade, proved to be well-known pathogens. This concurs with Diachun and Valleau's hypothesis (2) that perhaps some pathogenic fungi, in addition to the bacterial pathogens that they studied, can grow in association with roots of plants unrelated to the suscept. Nine isolates of *Verticillium albo-atrum* from nightshade roots were highly pathogenic to Lassen strawberry, but not to Pearson tomato, and 3 isolates of *Pythium ultimum* caused preemergence damping-off of Pearson tomato. Eight isolates of *Pyrenochaeta terrestris* caused pink root of

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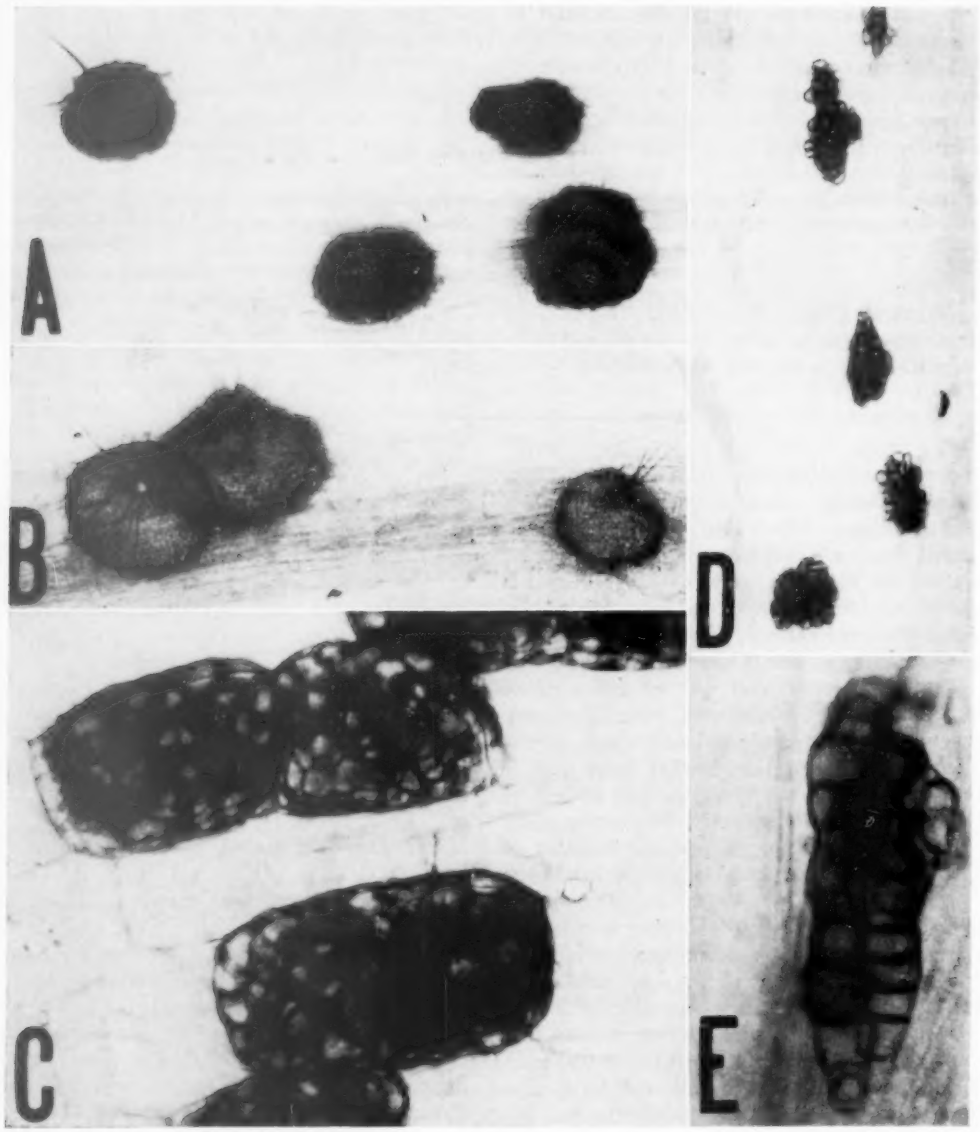

FIG. 1. Fungus structures photographed as they appeared upon removal of nightshade (except E, which was tomato) roots from sand cultures. A) Setose sclerotia of *Colletotrichum atramentarium*, × 115. B) Pycnidia of a Phoma, × 115. C) Resting structures of a gray sterile fungus, × 1100. D) Microsclerotia of *Verticillium albo-atrum*, × 290. E) Resting structure on *Pyrenochaeta terrestris*, × 1500.

white onion seedlings and also infected strawberry roots. The gray sterile fungus (Fig. 1, C) was pathogenic to strawberry roots, readily invading outer cortical cells and killing small rootlets (7). Two isolates of Stemphylium did not infect carrot seedlings; they probably were not *S. radicinum* (Meier, Drechs., & Eddy) Neerg. of carrot (4).

This technique has been used successfully in studies of the root fungi of some other plants, especially of tomato, Malva, various winter volunteer grasses, vetch, and alfalfa. For strawberry, the technique is not well adapted because roots usually darken after a short incubation.

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PHYTOPATHOLOGICAL NOTES

Seed Transmission of the Tomato-Ringspot Virus in the Lincoln Variety of Soybeans. Robert P. Kahn. Seed transmission of the tobacco-ringspot virus has been demonstrated in tobacco,[1] petunia,[2] and soybean[3] but apparently there have been no reports of seed transmission of the tomato-ringspot virus. These 2 viruses induce similar symptoms in inoculated soybean of the Lincoln variety,[4] but they may be differentiated on the basis of cross-immunity tests,[5] serologic reaction,[5] and particle size and shape.[6] The investigations reported herein were made to determine whether the tomato-ringspot virus is seed-borne. The tobacco-ringspot virus was tested in parallel series as a check on methods.

Tomato-ringspot virus was obtained from W. C. Price and tobacco-ringspot virus from W. B. Allington. Cross-protection tests[5] were used to differentiate the viruses.

Seeds were harvested from 3 sets of greenhouse-grown soybean plants of the Lincoln variety. One set had been inoculated in the primary leaf stage with tobacco-ringspot virus and a second with the tomato-ringspot virus. The third set comprised noninoculated virus-free plants. At the end of approximately 3 months, the virus-infected plants were still in an active vegetative stage and showed typical symptoms.[4] Seeds were collected from the few pods that matured on virus-infected plants and from several of the pods on virus-free plants. These were sown in the greenhouse during the winter months. Emergence was 32 per cent for seed from plants infected with tomato-ringspot virus, 44 per cent from plants infected with tobacco-ringspot virus, and 86 per cent for seed from virus-free plants.

All 3-week-old seedlings of these plantings were observed for symptoms and assayed for virus by rubbing Celite-dusted primary leaves of cowpea (*Vigna sinensis* Endl. var. Black) with juice expressed from leaves of the seedlings. The tomato-ringspot virus was transmitted to 76 per cent of the 34 seedlings. The tobacco-ringspot virus was transmitted to 82 per cent of 22 seedlings. All plants that were shown by the bioassay to contain virus showed typical bud-blight symptoms at maturity. These results confirm a previous finding of seed transmission of the tobacco-ringspot virus[3] and represent the first report of seed transmission of the tomato-ringspot virus. — Camp Detrick, Frederick, Maryland.

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Development of Conidia and Sclerotia of the Ergot Fungus on Inoculated Rye Seedlings.[1] Ralph W. Lewis. Cherewick[2] concluded from his inoculation tests that the ergot fungus (*Claviceps purpurea* (Fr.) Tul.) "may develop not only upon young ovaries but on any physiologically young tissue of wheat and barley plants." Stoll and Brack[3] had demonstrated previously that the meristem above the nodes in rye could be attacked by this fungus and that sclerotia would develop there. This suggested that seedlings of rye also might be susceptible. Seeds were germinated on moist paper in pans with metal covers. After inoculation they were placed on moist paper in pans with metal covers and allowed to grow at room temperature. Many hundreds of inoculations have been carried out successfully.

Seedlings were inoculated in a number of different ways. Inoculations were successful if spores were

[1] Paper No. 76 from the Department of Natural Science, Michigan State University. This work was supported by a grant from Eli Lilly and Company.

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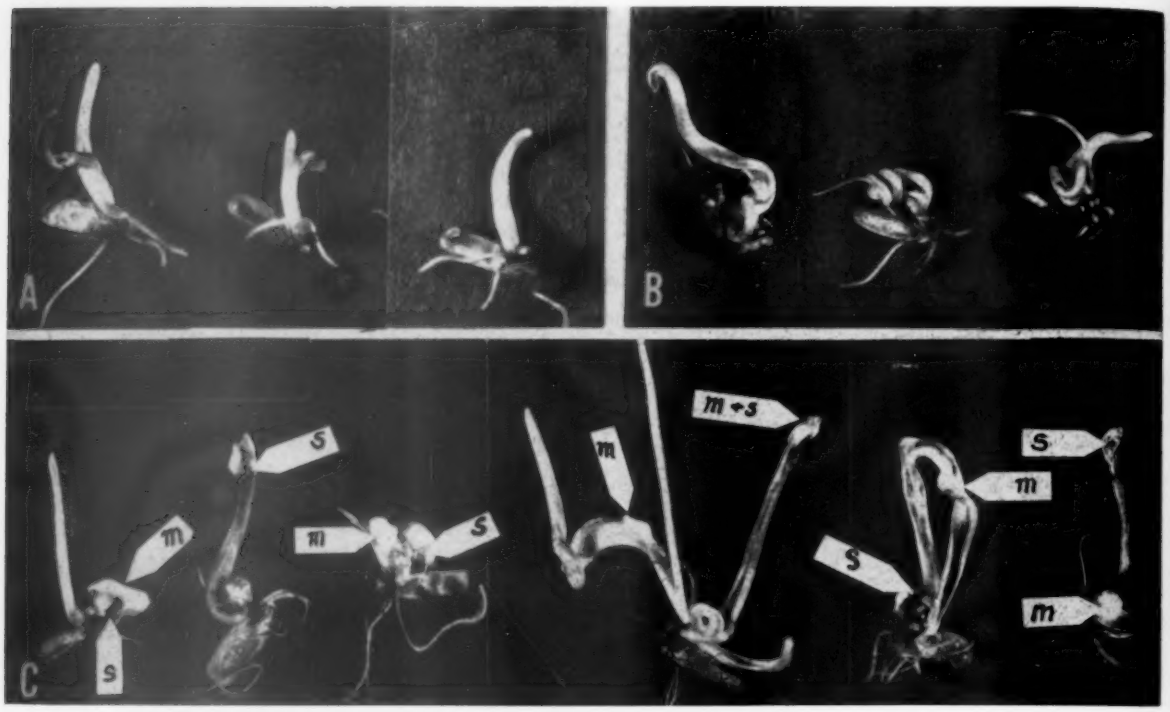

FIG. 1. Seedlings of 4 n Rosen rye inoculated with ergot fungus. A) Seedlings injected with sterile distilled water. B) Diseased seedlings 2–3 days after inoculation. Note curling of the shoots. C) Diseased seedlings 10–14 days after inoculation. The *m* arrows point to white mycelium; the *s* arrows point to black sclerotia.

placed in contact with meristematic tissue. This was done by placing the spores in slits, by cutting the shoot off 1–2 mm above the seed and placing spores on the cut surface, or by use of a hypodermic needle. The last method was most successful. A hypodermic syringe was mounted on a block of wood with a micrometer head in position to push the plunger. A spore suspension was prepared from a 14- to 18-day-old potato-dextrose agar slant culture by adding 5 ml of sterile water and rotating the tube rapidly between the hands until a cloudy suspension of spores was formed. This was taken up in a sterile tuberculin syringe with a No. 26 needle. The syringe was attached to its mounting block and adjusted so that the suspension could be fed through the needle by turning the micrometer head.

A seedling with a coleoptile 4–5 mm long was slit about 3 mm from the tip downward with the flattened needle-end. The needle-end was then gently pushed down through the slit into the center of the stem tip and rotated through ¼ turn to enlarge the space within the meristematic regions. A small drop of spore suspension (0.0008 ml) was then forced into the seedling by turning the micrometer screw 0.002 in. This amount of suspension will usually stay within the slit shoot. The drop is large enough to be seen, permitting a visual check on the inoculation. Care must be taken that the needle does not become plugged with tissue during the inoculations.

Variables affecting the percentage of infection have not yet been fully explored, but with 1 isolate of the fungus 30 per cent of the 2 n Rosen rye seedlings were infected as compared with 60 per cent infection in 4 n Rosen rye. (In the field, 4 n rye is much more susceptible than 2 n). Yorkwin wheat seedlings only rarely became infected.

Seedlings were considered to be infected only if the white cottony mycelium was visible to the naked eye. An infected seedling, however, may be curled in an unnatural manner so that infection often can be recognized before the mycelium is visible (Fig. 1, B). Mycelium usually is evident in 4–7 days, depending upon temperature and other factors (Fig. 1, C). Occasionally infection may not become evident until after 10 days.

One or 2 days after the mycelium is visible on a shoot, conidiophores and conidia are present. Both are similar in appearance to those produced in nature except that the palisade of conidiophores is not so dense and no honey-dew appears to be present.

Small sclerotia develop on some infected seedlings. They are usually embedded in a ball of white mycelium and become visible only if rubbed gently. Microscopic examination shows the sclerotia to consist of pseudoparenchymatous tissue typical of natural sclerotia. The outer wall is black and brittle (Fig. 1, C).—Department of Natural Science, Michigan State University, East Lansing, Michigan.

Frost Cracks on Oak. CHARLES L. FERGUS[1]. Frost cracks, radial splitting of tree trunks caused by sudden pronounced temperature drops, are commonly found in the northern hardwood forests. Little specific information is available as to their frequency of occurrence on hardwoods, other than the report of Kienholz and Bidwell[2]. Thus no valid estimates of their importance may be made. Observations about their occurrence on oaks in a central Pennsylvania woodlot were made to provide information about their importance.

The oaks examined were in a 10-acre oak-hickory type woodlot, consisting mostly of overmature trees that were approximately even-aged. The stand was very open and was exposed to open areas on the south and north sides. This woodlot contained a total of 969 trees, 577 of which were oaks. Seventy-two per cent of the oaks were dominant and codominant.

Data on frost cracks were taken only on *Quercus alba* L., white, and *Q. coccinea* Muench., scarlet, the only oak species present. Seventy-three, or 13 per cent, of the oaks had frost cracks. Thirty-three per cent of the trees with cracks had more than 1; 4 trees had 3 cracks, and 20 trees had 2. Cracks occurred in 17.5 and 22.3 per cent of the white and scarlet oaks, respectively. None of the white oaks had more than 1 crack.

Cracks were more frequent on large trees (94.7 per cent of the cracked trees had a diameter at breast height greater than 10 in.) than on smaller ones. A comparatively large proportion of the dominant trees had frost cracks. The 2 species combined showed the following percentages of cracked trees in each crown class: dominant, 19 per cent; codominant, 10 per cent; intermediate, 4 per cent; and suppressed, 5 per cent.

The cracks occurred at different heights on the bole and varied in length. Height was estimated from ground level to the lowest point of the crack. The lowest point of the crack occurred on the first 8-ft. log of all but 1, although many extended above it. The lowest point of 90 per cent of the cracks occurred within 4 ft. of the ground.

The cracks ranged in length from 6 in. to 21 ft. Sixty per cent of all cracks were 2 ft. or less in length. The longest were found on scarlet oaks. The average length of all cracks was 2.8 ft. This agrees closely with the observations of Kienholz and Bidwell.[2]

Stone[3] reported that almost all frost cracks occurred on the side of the tree exposed to the sun, generally a southern exposure. Kienholz and Bidwell[2], however, found that the greatest number faced the south, west, or north. In the present study, 53 per cent of the cracks faced north, northeast, or northwest; only 34 per cent faced south, southeast, or southwest. Some cracks were found facing all compass points. Thirty-six per cent of the trees with cracks were found on the exposed edges of the woodlot.

The cracks were healed on practically all trees, and 23 per cent of the cracks on scarlet oaks showed extensive callous growth called "frost ribs". On 1 tree, the rib extended 5 in. from the trunk and was 21 ft. long. No ribs were observed on white oaks. According to Boyce[4], such ribs indicate repeated opening of the crack by cold or by strains induced by wind, with intervening healing activity.

The importance of frost cracks probably varies considerably with the species concerned. Baxter[5] reported severe damage, often in the form of spiral cracks, to Norway spruce in Michigan plantations. No spiral cracks were observed in the oaks described here. Since most of the cracks occurred on the bole close to the ground and extended an average of 2.8 ft., the cull is small. The efficiency of log utilization by the sawyer and the possible presence and extent of associated decay, however, would determine the exact losses incurred.

Although many cracks heal over, the defects in the logs remain. Furthermore, healed cracks may reopen thus providing avenues of entrance for fungi that cause wood decay, wilt, or cankers.—Department of Botany and Plant Pathology, Pennsylvania State University, University Park, Pennsylvania.

Survival of Xanthomonas pelargonii in Soil. DONALD E. MUNNECKE. In a previous report,[1] data were presented on the survival of *Xanthomonas pelargonii* (Brown) Starr & Burk. in fallow soil for periods up to 3 months. The final results of the experiment are given here.

A field of heavy loam soil (Yolo series) was infested by plowing under geranium plants that had been inoculated with pure cultures of *X. pelargonii.* Immediately after plowing and at intervals of 1, 3, 6, and 12 months, 15 or 18 rooted cuttings of *Pelargonium hortorum* Bailey (Variety Radio Red) were planted in each of 4 randomized blocks in the field. The entire plot was irrigated with overhead sprinklers to facilitate rotting of the plowed-under plant parts, even though some blocks were not planted for 12 months. The blocks were spaced 3 ft. apart to minimize contamination by irrigation and rain water. Water was drained so that there was no run-off from one block to another, and care was taken to prevent movement of soil from one block to another in cultural operations. Disease ratings of each plant in each block

[1] Contribution No. 198 from the Department of Botany and Plant Pathology. Authorized for publication on September 8, 1955 as paper No. 2005 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

[2] Kienholz, R., and C. B. Bidwell. 1938. A survey of diseases and defects in Connecticut forests. Conn. Agr. Exp. Sta. Bul. 412, p. 493-559.

[3] Stone, G. E. 1912. Frost cracks. Twenty-fourth Ann. Rpt. Mass. Agr. Exp. Sta., p. 110-114.

[4] Boyce, J. S. 1948. Forest pathology. Ed. 2. McGraw-Hill. New York. 550 p.

[5] Baxter, D. V. 1952. Pathology in forest practice. Ed. 2. John Wiley and Sons. New York. 601 p.

[1] Munnecke, D. E. 1954. Bacterial stem rot and leaf spot of Pelargonium. Phytopathology 44: 627-632.

were made at monthly intervals on the basis of symptoms, and doubtful cases were verified by inoculation of healthy Radio Red plants in the greenhouse with an infusion from the suspect plant. The chance of secondary infection obscuring the results was minimized by removing the diseased plants as soon as they were definitely diagnosed. Healthy cuttings were planted in noninfested portions of the field as controls for each series.

The percentage of infected plants planted after delays of 1 day, and 1, 3, or 12 months were 100, 20, 11, and 0, respectively. Although 11 per cent of the plants in the 3-month series were diseased, the infected plants were confined to portions of 2 rows, indicating that small pockets of undecayed plant debris were probably present. The assumption that undecayed debris was present in small pockets in the field is difficult to prove; however, it is known from greenhouse experiments that the pathogen cannot survive in soil in pots after the plant debris is decomposed. In addition, there was no noticeable undecayed debris in the soil when the 3-month series was planted. Unfortunately, contaminated cuttings were used in the 6-month series, and the data had to be discarded. Plants of the 1-year series were observed for 18 months and showed no symptoms of disease during this period.

From these data and those reported previously,[1] it is clear that the bacteria have slight survival ability in moist soil, although 100 per cent loss may occur if cuttings are planted immediately in infested soil. Survival depends upon the rate of decay of the infected plant parts; probably 6 months is the limit of survival for the pathogen.—Department of Plant Pathology, University of California, Los Angeles.

A Simple Method for Identifying Snow Mold Damage on Turf Grasses.[1] J. B. Lebeau and M. W. Cormack.[2] In central Alberta, snow mold is caused by an unidentified low-temperature basidiomycete. Dead patches in early spring in golf courses, bowling greens, and lawns are often caused by this pathogen; however, similar damage to turf grasses may result from desiccation, trampling, and frost injury. A simple method for distinguishing snow mold from these other types of injury seemed desirable. The synthesis of HCN by the basidiomycete has been demonstrated under artificial conditions.[3] The production of this gas by the fungus suggested a quick method for identifying the disease. It was considered that chemical tests that established the presence of HCN in turf samples would differentiate between damage attributable to the snow mold pathogen and that caused by the other factors noted above.

Table 1.—*Concentration of HCN recovered from samples of turf grasses infected by the low-temperature basidiomycete at Edmonton and Calgary, taken at different times during the spring of 1955*

Turf grass	Date of sampling	Location	Average concentration HCN[a]
			ppm
Agrostis tenuis Sibth.	April 1	Edmonton	96
	April 7	Edmonton	26
Poa pratensis L.	April 7	Edmonton	30
A. palustris Huds.	April 15	Calgary	36
P. pratensis	April 15	Calgary	22
Festuca rubra L.	April 18	Edmonton	10
A. tenuis	April 18	Edmonton	5
	May 1	Edmonton	0
P. pratensis	May 1	Edmonton	0
Controls[b]			0

[a] HCN expressed on oven-dried weight of turf samples.
[b] Control samples taken at both locations at each date of sampling.

Samples of damaged turf were obtained during April and May from 2 golf courses in Edmonton and 1 in Calgary, and also from several lawns in Edmonton. Control samples were removed from healthy turf, from pathways where killing was attributed to trampling, and from turf of *Poa annua* L., which does not overwinter in this climate. Plugs about 3 in. in diameter were cut from the turf and removed to the laboratory where they were steam-distilled into a 2 per cent KOH solution. Hydrogen cyanide determinations were made by the method outlined by Robbie.[4] In an attempt to isolate the fungus, small pieces of turf from each sample were also plated on potato-sucrose agar and incubated at 2°C.

All samples taken in April from areas containing infected plants gave positive tests for HCN, whereas the controls showed no trace of cyanide (Table 1). Samples taken in early April gave stronger tests for HCN than did those removed later in the month, whereas those taken in May failed to give positive tests. Hydrogen cyanide apparently was not fixed in the plant tissue or soil and was volatilized as soon as the temperature rose in the spring. Isolation results confirmed the presence of the low-temperature basidiomycete in all of the samples containing HCN.

Since the low-temperature basidiomycete also causes winter crown rot of alfalfa and other forage crops in Western Canada, this technique may also be applied to these crops early in the spring and should eliminate difficult and time-consuming isolations to identify the disease.—Plant Pathology Laboratories, Edmonton and Lethbridge, Alberta, Canada.

[1] Contribution No. 1503 from the Botany and Plant Pathology Division, Science Service, Canada Department of Agriculture, Ottawa, Ontario.

[2] Associate Plant Pathologist, Edmonton, and Officer-in-Charge, Lethbridge, respectively.

[3] Lebeau, J. B., and J. D. Gickson. 1953. Preliminary report on production of hydrogen cyanide by a snow-mold pathogen. Phytopathology 43: 581-582.

[4] Robbie, W. A. 1944. An improved phenolphthalin technique for the microdetermination of cyanide. Arch. Biochem. 5: 49-58.